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PROVISIONAL APPLICATION FOR PATENT COVER SHEET

This is a request for filing a PROVISIONAL APPLICATION FOR PATENT under 37 CFR 1.53(c).

INVENTOR(S)

Given Name (first and middle [if any])	Family Name or Surname	Residence (City and either State or Foreign Country)
Susanne	Binder	Vienna, Austria, Europe
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☐ *Additional inventors are being named on the ____ separately numbered sheets attached hereto*

TITLE OF THE INVENTION (280 characters max)

RETINAL PIGMENT EPITHELIAL CELL CULTURES ON AMNIOTIC MEMBRANE AND TRANSPLANTATION

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ENCLOSED APPLICATION PARTS *(check all that apply)*

☒ Specification *Number of Pages* 46 — ☐ CD(s), Number ____

☒ Drawing(s) *Number of Sheets* 8 — ☒ Other (specify) Attachment (47-55)

☒ Application Data Sheet. See 37 CFR 1.76

METHOD OF PAYMENT OF FILING FEES FOR THIS PROVISIONAL APPLICATION FOR PATENT *(check one)*

☒ Applicant claims small entity status. See 37 CFR 1.27.

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Respectfully submitted,

SIGNATURE Jacqueline M. Arendt — Date 10/04/02

TYPED or PRINTED NAME Jacqueline M. Arendt, Esq. — REGISTRATION NO. *(if appropriate)* 43,474

TELEPHONE 518-452-5600 — Docket Number: 2119.003P

USE ONLY FOR FILING A PROVISIONAL APPLICATION FOR PATENT

CERTIFICATE OF MAILING BY EXPRESS MAIL@

Applicant: Susanne Binder and Scheffer C.G. Tseng

Title: RETINAL PIGMENT EPITHELIAL CELL CULTURES ON AMNIOTIC MEMBRANE AND TRANSPLANTATION

Attorney Docket No.: 2119.003P

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Inventors: Susanne Binder and Scheffer C.G. Tseng

Attorney's Docket No.: 2119.003P

RETINAL PIGMENT EPITHELIAL CELL CULTURES ON AMNIOTIC MEMBRANE AND TRANSPLANTATION

BACKGROUND OF THE INVENTION

The retina is a multi-layered nervous tissue where light energy is converted into nerve impulses. The outermost layer of the retina, closest to the front of the eye, is a layer of neurons that includes ganglion cells. Behind the ganglion cells is a layer of integrating neurons, and behind the integrating neurons is a layer of photoreceptor cells, called rods and cones. Photoreception in rods and cones begins with absorption of light by a pigment in the cells, the absorbed light causing a receptor potential.

Forming an intimate structural and functional relationship with the photoreceptor cells is the retinal pigment epithelium, a monolayer of specialized, cuboidal cells located immediately behind the retina. The retinal pigment epithelial (RPE) cells provide support for the photoreceptor cells and carry on important physiological functions, including solute transport, phagocytosis and digestion of discarded outer segments of membranes shed from photoreceptor cells, and drug detoxication.

The RPE cells rest on a specialized basement membrane, called the Bruch's membrane, a membrane 1 to 5 microns in thickness and composed of collagen, laminin and other molecules.

Underlying the RPE cells is the choriocapillaris of the choroid tissue. The choriocapillaris contains the vasculature to provide nutrients and remove metabolic by-products from the retina. Underlying the choroid tissue is the sclera.

It is believed that failure of the RPE cells to properly perform their functions alters the extracellular environment for photoreceptor cells, and leads to the eventual degeneration and loss of photoreceptor cells. Dysfunction of RPE contributes to the pathogenesis of a variety of sight-threatening diseases including age-related macular degeneration (ARMD) 1, serous retinal detachment 2, and such genetic diseases as gyrate atrophy 3 and choroideremia 4.

Age-related Macular Degeneration

ARMD is the leading cause of visual impairment in western countries and is believed to be caused by progressive deterioration of RPE, Bruch's membrane, and the choriocapillaris, which leads to subsequent damage to the photoreceptor cells. In ARMD, the RPE cells are dysfunctional. In one form of ARMD, degeneration of the RPE cells is followed by atrophy of the choriocapillaris. In another form, the Bruch's membrane is altered and degraded by invasion of choroid neovascular membrane (CNV) into the subretinal space, leading to hemorrhage in the subretinal space, and scarring, with possible further damage to both RPE and photoreceptors.

Although (CNV) 5 into the subepithelial and/or subretinal space can be treated with laser photocoagulation, if neovascularization is subfoveal, the results are poor 6. Surgical removal of CNV membranes seldom leads to improvement of vision or halts the progression of ARMD 7. The poor results may be due to inadvertent removal of RPE during the CNV removal 8, the failure of RPE to repopulate, the progressive

enlargement of choriocapillaris atrophy following submacular surgery 9, and photoreceptor loss.

Problems with Prior Art Methods of RPE Transplantation

Only limited success in restoring vision using current methods of RPE transplantation has been achieved with either autologous or allogeneic sources (experimental 6;10 and clinical 11-14). In experimental animals, in particular in the Royal College of Surgeons (RCS) Rat model of macular degeneration 15-19, RPE transplantation has been used to rescue photoreceptors, preserve choriocapillaris, and prevent CNV. In the case of allogeneic RPE transplantation, one obvious reason to explain the failure is allograft rejection 13;20. However, in the case of autologous RPE transplantation, the failure to restore vision may be due to the failure of transplanted RPE to repopulate the diseased site or to function *in vivo*. The failure of the RPE to grow or to function may be due to damage to the Bruch's membrane.

The Bruch's Membrane

There is evidence that the integrity of Bruch's membrane is crucial for RPE repopulation and subsequent functions. For example, surgical removal of RPE without damage to Bruch's membrane results in partial regeneration of the RPE monolayer in the non-human primate and domestic pig with the preservation of the underlying choriocapillaris and the overlying photoreceptors 21-23. In contrast, abrasive debridement causes more damage to Bruch's membrane, leads to incomplete repopulation of RPE, choriocapillaris atrophy, and outer segment retinal degeneration 24. Experimental transplantation of cultured human RPE to Bruch's membrane of the owl's monkey eye results in normal attachment, viability, and expressing junctions and morphological polarity 25. Autologous transplantation of RPE onto an abrasively

debrided Bruch's membrane decreases choriocapillaris atrophy and photoreceptor loss in rabbits 26.

In the case of human patients with ARMD, the failure of restoring RPE function in transplanted human autologous RPE may be at least partially due to the altering of Bruch's membrane intrinsically caused by ARMD 27 and damaged by surgical removal of CNV membrane 23;24.

The current method of RPE transplantation, subretinal injection of an RPE cell suspension, achieves a limited success. There are many problems associated with this method, including a resulting subretinal fibrosis and the formation of multiple layers of RPE 6. These problems may be due to lack of restoration of *in vivo* (normal) epithelial phenotype and function. To date, no advance has been made in restoring Bruch's membrane in the surgical treatment of ARMD.

Immunological Aspects of RPE Transplantation

Although the eye as a part of the central nervous system has characteristics of an immunologically privileged site, it has been demonstrated that RPE transplants sensitized their recipients to both alloantigens and to RPE-specific autoantigens. Both are considered potential barriers to successful transplantation, and would make immune suppression regimens necessary 28. It was also demonstrated that the immunological response is most likely related to the amount of transplanted cells and that the response increases with time. RPE allografts in the RCS rat were not rejected for up to one year.

Problems with Prior Art Substrates and Methods of Culturing RPE

Substrates that have been used for this purpose include plastic 31, cross-linked collagen 32, gelatin 1, fibrinogen 2, poly-L-lactice acid (PLLA) 3, PLLA/PLGA (poly-DL-lactic-co-glycolic acid) film 5-6, hydrogel 7, and basement membrane-containing anterior lens capsule 7. There are many disadvantages associated with each of the prior

art substrates used for culturing RPE cells for transplantation, and a number of problems remain unsolved.

Impermeable substance

One attempt at RPE transplantation utilized RPE cells isolated from either the whole eye or from a biopsy with Dispase® (GodoShusei Co., Ltd., Tokyo, Japan), and seeded on an impermeable substrate such as the plastic dish 31. These cells were prepared as a dissociated cell suspension 6;10 or as a patch derived from fetus 11 before transplantation. These cells did not fully retain their epithelial morphology. Furthermore, pigmentation of melanolipofuscin granules rapidly disappears on plastic cultures 33.

Porous support (cross-linked collagen, collagen, gelatin, fibrinogen, PLLA/PLGA, hydrogels, CNV membranes, lens capsule)

Cross-linked collagen, when used for transplantation, is damaging to the retina due to its thickness, poor permeability and inability to degrade 32. Although human RPE cells 34 seeded on collagen membrane produced a monolayer of cells that exhibited a measurable transepithelial resistance and electrical potential 35, the cells did not achieve the *in vivo* state of development and function.

Gelatin is used as an embedding medium, but not as a substrate for attachment. Fibrinogen and PLLA microspheres are also not suitable for transplanting RPE as a single sheet when transplanted to the subretinal space 2; 3. PLLA/PLGA films do provide the RPE monolayer sheet for transplantation, but *in vitro* cultures of human fetal RPE cells grown on these supports do not show pigmentation (melanogenesis) 5; 6. Hydrogel also provides the RPE monolayer sheet for transplantation, but the resultant cell density and the cell tight junction determined by expression of ZO-1, is relatively low 7.

Human RPE cells have also been cultured on surgically excised CNV membranes from ARMD patients, but the culture forms multiple layers 36.

Although lens capsule is a basement membrane –containing, natural material, it is not an ideal substrate for RPE culture and transplantation. Anterior lens capsule has been used to grow RPE 7; 8 and IPE 8, and to transplant RPE and IPE 8 with lens capsule to the subretinal space. Both hydrogel and lens capsule, when used as substrates for RPE cultures, do not allow pigmentation to form (or melanogenesis) by RPE cells in culture 7; 8.

The inventors of the present subject matter attempted to use the lens capsule as an autologous substrate for RPE/IPE transplants. However, the tendency of the capsule to curl made this technique impracticable. The idea to use the posterior capsule, because it is thinner, was also abandoned, for a number of reasons. First, the posterior capsule is difficult to obtain during surgery, without putting the patient at a high risk. Secondly, no absorption or slow absorption of the lens capsule material might inhibit the survival of the transplanted RPE cells because of insufficient contact of the cells with the Bruch's membrane and/or choriocapillaris.

Recently Bilbao *et al*, 37 disclosed the use of PLGA, coated on one side of a lens capsule to prevent curling and to facilitate its use for subretinal release. However, histological studies showed not only that the PLGA had completely dissolved after 4 weeks, but also that the overlying retinal layers were disrupted, the disruption accompanied by a large amount of cell infiltration.

Cryoprecipitate from blood donors was also tested as a possible autologous substrate for human fetal retinal pigment epithelium by Farrokh- Siar et al in 1999 (38). Dutt *et al*, 39 used several substrates for culture of HPE cell line 0041: extracts from placenta and amnion; Matrigel® (Collaborative Biomedical Products. Inc., Bedford, MA), a commercially available basement membrane matrix; dishes coated with extracellular matrix secreted by endothelial cells (ECM); dishes coated with collagen IV and/or laminin; dishes coated with collagen I and /or fibronectin. Although deeply pigmented, cells grown on Matrigel® looked like fibroblasts.

As described above, problems that remain to be solved include, for example, maintenance of the morphology of the RPE phenotype in cultured and transplanted RPE cells, creation of a uniform monolayer of autologous RPE on a biocompatible substrate, improvement of the transplant technique to better cover the defect, overcoming immune rejection of RPE transplants due to both alloantigens and RPE-specific auto-antigens, and prevention of subretinal fibrosis following RPE transplantation.

Amniotic membrane is a biological membrane that lines the inner surface of the amniotic cavity and comprises a simple, cuboidal epithelium, a thick basement membrane, and an avascular mesenchymal layer containing hyaluronic acid. Amniotic membrane transplantation has been used for ocular surface reconstruction in the treatment of acute chemical and thermal burns of corneal tissue 53.

Overall, the medical need for a method of culturing RPE cells suitable for transplantation to the subretinal space, a suitable RPE transplant composite, and a method of transplanting RPE cells to the subretinal space, has not been met.

SUMMARY OF THE INVENTION

The present invention relates to the discovery that cryopreserved amniotic membrane, when appropriately procured and processed, can be used for the culturing of RPE cells thereon, and as a surgical graft for replacement of RPE, Bruch's membrane, and underlying choriocapillaris; and that the graft does not elicit immunological reactions. As such, the invention relates, in one aspect, to compositions for implantation in the subretinal space of an eye of a patient in need thereof, the composition including amniotic membrane, and a plurality of retinal pigment epithelial cells or retinal pigment epithelial equivalent cells present at the amniotic membrane. In one embodiment of the invention, the amniotic membrane present in the composite is human amniotic membrane. The amniotic membrane may be intact, epithelially denuded, or treated on one side, for example to thin or remove one side. In another embodiment, the amniotic membrane is reshaped by laser ablation to remove the

stromal side or to thin the basement membrane side. In yet another embodiment mesenchymal cells are added to the stromal side. The invention, *inter alia,* comprises the following, alone or in combination.

Another embodiment includes a kit comprising amniotic membrane; a plurality of retinal pigment epithelial cells or retinal pigment epithelial equivalent cells present at the amniotic membrane; a buffer medium or a culture medium; and optionally, instructions for simultaneous, separate, or sequential use of at least one component of the kit for treating a retinal disease. In a particular embodiment, the amniotic membrane included in the kit is human amniotic membrane.

The present invention also relates, in another aspect, to a method of forming a composite for implantation in the subretinal space of an eye of a host in need thereof, the method including the steps of applying at least one retinal pigment epithelial cell or retinal pigment epithelial equivalent cell to an amniotic membrane; and culturing the retinal pigment epithelial cell or retinal pigment epithelial equivalent cell on the membrane. The amniotic membrane used in one embodiment may be human.

The present invention also relates, in yet another aspect, to a method of inducing an excised or cultured retinal pigment epithelial cell or retinal pigment epithelial equivalent cell to express or to maintain the phenotype of retinal pigment epithelial cells, the method including the steps of contacting amniotic membrane with the retinal pigment epithelial cell or retinal pigment epithelial equivalent cell; culturing the retinal pigment epithelial cell or retinal pigment epithelial equivalent cell on said membrane under conditions suitable for growth for a period of time sufficient to produce a plurality of cultured cells; and/or either contacting the cultured cells with an effective amount of an agent that raises the intracellular calcium ion concentration to a level sufficient to induce or maintain the phenotype of retinal pigment epithelial cells; or exposing the membrane including the cultured cells to an air-fluid interface for a period of time sufficient to induce or maintain the phenotype of retinal pigment epithelial cells. The amniotic membrane used in an embodiment may be human.

In a particular embodiment, both the step of contacting the cultured cells with an agent that increases the intracellular calcium ion concentration, and the step of exposing the membrane including the cultured cells to an air-fluid interface to induce or maintain the phenotype of retinal pigment epithelial cells, are performed sequentially or essentially simultaneously.

The present invention also relates, in yet another aspect, to a method of using amniotic membrane to promote the growth and the differentiation of at least one retinal pigment epithelial equivalent cell to a plurality of cells that express the phenotype of retinal pigment epithelial cells. In a particular embodiment, the amniotic membrane is human.

Another embodiment of the invention is a method of delivering a plurality of retinal pigment epithelial cells to a target site in a subretinal space in an individual in need thereof, including the steps of forming at least one hole in a retina of the individual, or at least partially detaching the retina to access the subretinal space; inserting through the hole a composite including amniotic membrane and the retinal pigment epithelial cells present at the membrane; and positioning the composite at the target site.

The invention also relates to a method for treating a retinal disease, the method including inserting in a subretinal space of a patient in need thereof a composite comprising amniotic membrane, for example, human amniotic membrane, and a plurality of retinal pigment epithelial cells present at the membrane. The RPE cells may be cultured on the membrane according to a method of the invention. An example of a retinal disease that is treated according to an embodiment of the invention is age-related macular degeneration. Other examples include retinal detachment, gyrate atrophy, and choroideremia.

Another aspect of the invention is the use of amniotic membrane, which in one embodiment is human in origin, and at least one retinal pigment epithelial cell, for the manufacture of a composition for treatment of a retinal disease in a patient suffering from, or at risk of developing the disease.

The invention also relates to the use of human amniotic membrane to promote growth and differentiation of at least one retinal pigment epithelial equivalent cell to a plurality of cells that express the phenotype of retinal pigment epithelial cells.

Yet another aspect of the invention is the use of amniotic membrane in transplanting retinal pigment epithelial cells or iris pigment epithelial cells to a subretinal space, to prevent or decrease a sensitizing of a recipient to alloantigens and to retinal pigment epithelial-specific autoantigens. In a particular embodiment, the amniotic membrane used in transplanting RPE cells or IPE cells to a subretinal space, to prevent or decrease a sensitizing of a recipient to alloantigens and to retinal pigment epithelial-specific autoantigens is human in origin.

The invention also relates to the use of amniotic membrane, including human amniotic membrane, to inhibit fibrosis following transplantation of RPE cells or IPE cells to the subretinal space. Another embodiment of the invention is the use of human amniotic membrane and at least one RPE cell for the manufacture of a composition for treatment of a retinal disease in a patient suffering from, or at risk of developing the disease.

The use of amniotic membrane, processed and cryopreserved according to an embodiment of the invention, as a substrate for the culturing of RPE or RPE equivalent cells thereon, and as a surgical graft for substrate replacement and the transplanting of RPE cells in the subretinal space, exerts anti-inflammatory, anti-angiogenic, anti-fibrotic, and anti-scarring effects, and does not elicit immunological reactions to either alloantigens or RPE - specific auto-antigens. Further, amniotic membrane used according to an embodiment of the invention promotes growth and differentiation of RPE cells in culture, and maintenance of the morphological appearance of the RPE cells both in culture and following transplantation to the subretinal space. The composite and method of delivery of RPE cells to the subretinal space result in a monolayer of RPE cells on a substrate having a basement membrane.

BRIEF DESCRIPTION OF THE DRAWINGS

The foregoing and other objects, features and advantages of the invention will be apparent from the following more particular description of preferred embodiments of the invention, as illustrated in the accompanying drawings. The drawings are photographs of RPE cells at various stages of culture and under various conditions, as well as photographs of stained RPE *in vivo* rabbit retina. The figures help illustrate principles of the invention.

Fig. 1A is a photograph (10 x) of RPE cells on plastic culture after confluence in low calcium ion medium.

Fig. 1B is a photograph (10 x) of RPE cells on denuded human amniotic membrane (dAM) after confluence in low calcium ion medium.

Fig. 1C is a photograph (10 x) of RPE cells on intact human amniotic membrane (iAM) after confluence in low calcium ion medium.

Fig. 1D is a higher magnification (40 x) of Fig. 1C.

Fig. 2A and Fig. 2B are photographs (40 x) of the cell cultures in Fig. 1A and 1B, respectively, one week after the medium was switched to high calcium ion medium.

Fig. 2C is a photograph (10 x) of the cell culture in Fig. 1C one week after the medium was switched to high calcium ion medium.

Fig. 2D is a photograph (10 x) of the cell culture in Fig. 1D one week later, with the low calcium ion concentration maintained.

Fig. 3A is a photograph (10 x) of the RPE cell culture on plastic, low calcium ion, at confluence, with immunostaining by antibody to cytokeration 18 (CK 18).

Fig. 3B is a photograph (10 x) of the RPE cell culture of Fig. 3A, but after 1.5 weeks at high calcium ion concentration, with immunostaining by CK18.

Fig. 3C is a photograph (10 x) of the RPE cell culture of Fig. 2C (on IAM) after 1.5 weeks at high calcium ion concentration, with immunostaining by CK18.

Fig. 3D is a photograph (10 x) of the RPE culture on dAM after 1.5 weeks at high calcium ion concentration, with immunostaining by CK18.

Fig. 4A through Fig. 4D are photographs of RPE *in vivo* rabbit retina staining with RP65, the RPE located between the photoreceptors and choriocapillaris.

Fig. 5A is a photograph of the RPE cell culture on plastic, after 1.5 weeks on high calcium ion concentration, with RP65 staining.

Fig. 5B and 5C are photographs of RPE cell cultures on iAM (5B) and dAM (5C) after 1.5 weeks on high calcium ion concentration, with RP65 staining.

Fig. 5D and Fig. 5E are the RPE cell cultures shown in Fig. 5B and Fig. 5C, respectively, after 3.5 weeks at high calcium with RP65 staining.

Fig. 6A – D are photographs of RPE *in vivo* after Z0-1 staining.

Fig. 7A is a photograph (10 x) of an RPE cell culture at confluence, low calcium ion, with ZO-1 staining, on plastic.

Fig. 7B is a higher magnification (40 x) of Fig. 7A.

Fig. 7 C1 is a photograph (10 x) of an RPE cell culture at confluence, low calcium ion, with ZO-1 staining, on iAM.

Fig. 7 C2 is a higher magnification (40 x) of Fig. 7C1.

Fig 7 D is a photograph (10 x) of an RPE cell culture at confluence, and low calcium ion, with ZO-1 staining, on dAM.

DETAILED DESCRIPTION OF THE INVENTION

A description of preferred embodiments of the invention follows. It will be understood that the particular embodiments of the invention are shown by way of illustration and not as limitations of the invention. At the outset, the invention is described in its broadest overall aspects, with a more detailed description following. The features and other details of the compositions and methods of the invention will be further pointed out in the claims.

The inventors of the subject matter of the present invention attempted first to use the stromal side of amniotic membrane as a substrate on which to grow human umbilical vascular endothelial cells, but the cells would not grow on amniotic membrane. In fact, the endothelial cells underwent apoptosis. The inventors also used the stromal side of amniotic membrane as a substrate in an attempt to grow human polymorphonuclear leucocytes thereon, but the leucocytes would not grow on amniotic membrane.

The invention relates to the discovery that, under suitable conditions, RPE cells, RPE equivalent cells, and IPE cells will grow on appropriately procured and processed, cryopreserved amniotic membrane, for example, human amniotic membrane. Histologically, amniotic membrane comprises a thick basement membrane and an avascular stroma. The inventors have discovered that human amniotic membrane is an ideal extracellular matrix substrate to promote growth and differentiation of RPE equivalent cells and RPE cells in culture.

The RPE cells, RPE equivalent cells, and IPE cells grown on amniotic membrane, under suitable conditions, differentiate, tend to retain their morphological characteristics, and do not tend to de-differentiate. Further, the amniotic membrane with the cells present thereon can be used as a surgical graft to transplant RPE cells to the subretinal space, and for substrate replacement. The graft does not elicit immunological reactions, and can be used to treat retinal diseases.

Accordingly, the invention relates to the resulting composite, including amniotic membrane, and a plurality of RPE, RPE-equivalent cells, or IPE cells, and to a method of use of the composite as a surgical graft to transplant RPE to the subretinal space of a mammalian eye in order to treat retinal diseases such as age-related macular degeneration, retinal detachment, gyrate atrophy, and choroideremia.

RPE or RPE-equivalent Cells

The term, "RPE equivalent cells," as used herein, refers to cells that are derived from either retina, iris, adult stem cells or embryonic stem cells, and which either retain their normal phenotype or function; or which may have less than optimal function; or which have been induced *in vitro* or *in vivo* to differentiate into RPE cells.

According to an embodiment of the invention, the source of RPE cells can be from human or other mammals. According to another embodiment of the invention, the source of RPE cells can be either autologous (from the same individual as the recipient) or allogeneic (from a different individual from the recipient). For the latter, these cells can be either obtained from adult or fetal, cadaveric or living individuals, of which the latter can be HLA-matched or non-matched.

Further, the source of human RPE equivalent cells can be derived from retina or iris. The RPE cells, in one embodiment, comprise cells derived from neural retinal cells, for example, rod cells or cone cells. If derived from iris, the cells are termed iris pigment epithelial cells (IPE), and their function may be suboptimal. The source of human RPE equivalent cells can also be derived from RPE cells that have been immortalized by viral or non-viral agents but still retain normal phenotype or function.

The source of human RPE equivalent cells can also be derived from adult stem cells or embryonal stem cells, of which the differentiation into RPE has been induced *in vitro*. For the former, such adult stem cells can be obtained either autologously or allogeneically from various sites of the body, for example, from the peripheral blood or bone marrow.

The source of RPE equivalent cells can also be derived from other non-human species but bioengineered so that they become compatible with human cells. For example, a source of the retinal pigment epithelial equivalent cells used in a composite according to the invention may include at least one bioengineered cell induced *in vitro* to differentiate into a retinal pigment epithelial cell.

Means of Harvest of RPE Cells

If the source of RPE or IPE is autologous, the means of harvest will be surgical biopsy from the tissue site of retina or iris. Autologous RPE equivalent cells are derived from adult stem cells, and can be obtained from the site of interest, e.g., peripheral blood or bone marrow. If the source of RPE or IPE is allogeneic, either from a living individual, or cadaveric, they will be obtained form donated tissues, respectively. Other sources of allogenic RPE equivalent cells are well known to those of skill in the art of tissue culture and transplantation.

Harvest and Culturing of RPE or RPE equivalent Cells

The method of harvesting or isolating RPE or IPE cells is conventional and includes enzymatic digestion using collagenase or Dispase® solutions. The method of harvesting or isolating RPE equivalent cells induced from stem cells is performed *in vitro* using growth factors and inducible factors. After harvesting, these cells are cultured in a medium containing culturing supplement including serum and growth factors. The medium will comprise a low calcium ion concentration in the range of about 0.01mM to about 0.4 mM, preferably 0.1 mM. The expansion culture is performed on a culturing substrate of choice. According to an embodiment of the invention, the expansion culture is performed on cryopreserved amniotic membrane.

Method of Preparing Amniotic Membrane

Methods of preparing cryopreserved human amniotic membrane suitable for use in an embodiment of the invention are well known in the art and are described, for example in U.S. Patent Numbers 6,152,142 and 6,326,019 B1 to Tseng, the teachings of each of which are incorporated herein by reference in their entireties. Methods of

preservation of amniotic membrane are also described in WO 01/08716 A1, the teachings of which are incorporated herein by reference in their entirety.

Amniotic membrane suitable for use in an embodiment of the invention is obtained from mammalian placenta, especially human placenta, from which the chorion has been separated. The amniotic membrane used in an embodiment may also be derived, for example, from an equine, a bovine, or an alpaca source. Amniotic membrane suitable for use in an embodiment of the invention generally includes an epithelial layer, a basement membrane, and a stroma, the combination of the three layers preferably having an average total thickness of about 200 μm. Sheets of the amniotic membrane can be cut to size, mounted on filter paper, and stored in a storage solution. The storage solution comprises a culture medium and a hyperosmotic agent, wherein the hydration of the amniotic membrane is maintained. The membrane can be impregnated with therapeutic agents, prior to storage or prior to use.

For use in an embodiment of the invention, the amniotic membrane is either intact (i.e., without additional treatments) or epithelially denuded (i.e., by EDTA and mechanical means as reported previously 62) See, Grueterich M, Espana E, Tseng SCG., Connexin 43 expression and proliferation of human limbal epithelium on intact and denuded amniotic membrane, *Invest Ophthalmol Vis.Sci.*, 43:63-71 (2002), attached hereto and incorporated herein by reference in its entirety on the epithelial surface. The amniotic membrane is either intact or ablated to remove the stromal portion on the stromal surface. If the epithelially denuded membrane is to be used, the denuded membrane is prepared before being seeded with RPE or RPE equivalent cells in culture (see below). However, if the amniotic membrane stroma is to be thinned, the stroma should be ablated either before or after such culturing. The method of ablation can be laser-driven, for example, by excimer laser. In other embodiments, the amniotic membrane can be treated to enable the RPE cells to better adhere to the membrane. For example, the membrane can be treated to produce an electrical charge thereon.

Method of Culturing RPE or RPE equivalent Cells on Amniotic Membrane

A standard culturing method is used according to an embodiment of the invention. Cell seeding density can be varied depending on the surface area of amniotic membrane used. RPE or RPE equivalent cells are generally removed from a plastic substrate when they reach the subconfluent stage by conventional methods utilizing trypsin and/or EDTA. The isolated RPE or RPE equivalent cells are seeded on the amniotic membrane on the epithelial side, with the basement membrane either exposed or still covered by intact amniotic epithelial cells.

Methods of Inducing Epithelioid Phenotype in Culture of RPE on Amniotic Membrane

According to an embodiment of the method, the step of culturing the retinal pigment epithelial cell or retinal pigment epithelial equivalent cell on the membrane is continued until the cells reach confluence. Ideally, the number of retinal pigment epithelial cells present at the membrane is about 4000 cells per 1 mm^2. However, the ideal number will depend on the size of the defect to be covered with the transplanted composite. For example, between about 16,000 and about 20,000 cells with high vitality are needed to cover a $4mm^2$ defect.

One method according to the invention, of inducing epithelial phenotype from fibroblastic phenotype of RPE, is to elevate calcium concentration from low (for example, a range of about 0.01 to about 0.4 mM, preferably about 0.1 mM) to high in the range of about 0.5 to about 2.0 mM, preferably about 1.8 mM . The calcium ion concentration may be elevated, according to an embodiment, by adding a soluble calcium salt to the culture medium. Alternatively, an agent such as a calcium ionophore, which facilitates transport of calcium ion across the lipid barrier of the cell membrane by combining with the ion or by increasing the permeability of the barrier to the ion may be used to increase calcium ion concentration. Another embodiment includes an agent that increases intracellular calcium concentration by blocking the

export of calcium out of the cytoplasm. According to another embodiment, the amniotic membrane comprising RPE or RPE equivalent cells cultured thereon is exposed to air-fluid interface. A combination of both methods can also be employed.

The Composite Including RPE Cells or RPE Equivalent Cells on Amniotic Membrane

According to one embodiment, a composite includes intact human amniotic membrane comprising a basement membrane and a stroma. In another embodiment of the invention, the human amniotic membrane of the composite is epithelially denuded.

The invention also relates to a composite that further includes at least one pharmaceutically active molecule. In one embodiment of the invention, the pharmaceutically active molecule in the composite is one or more of the following: a growth factor, an enzyme, or a therapeutic drug.

A Kit for Treating Retinal Disease

Another embodiment includes a kit comprising amniotic membrane, which may be human amniotic membrane; a plurality of retinal pigment epithelial cells or retinal pigment epithelial equivalent cells present at the amniotic membrane; a buffer medium or a culture medium; and optionally, instructions for simultaneous, separate, or sequential use of at least one component of the kit for treating a retinal disease. In a particular embodiment, the kit further includes at least one pharmaceutically active agent. The agent may include growth factors, enzymes, and therapeutic drugs. The growth factor may include retinal pigment epithelium-derived growth factor and/or transforming growth factor-beta. The agent may include interleukin-10.

The agent may be present on the composite, or separately packaged, to be added to the composite or to the target tissue site in the subretinal space prior to implantation, or to be administered to the patient subsequent to transplantation.

The kit according to an embodiment may include retinal pigment epithelial equivalent cells that are bioengineered cells.

The kit according to an embodiment may include a composite formed of amniotic membrane and retinal pigment epithelial equivalent cells of autologous origin that have been previously harvested from the intended recipient, sent to a laboratory wherein the cells are cultured on amniotic membrane, and made into the composite.

Method of Transplant of RPE Cells to the Subretinal Space

The surgical procedure for transplant of RPE is similar to standard procedure for intraocular surgery.

The procedure comprises the following steps:

1) Pars plana vitrectomy to remove the posterior hyaloid;

2) The first retinotomy is performed temporally or nasally superior to the CNV membrane;

3) Using Ringer's solution, the submacular CNV membrane is gently hydrodissected and removed with a subretinal forceps. During this step, the intraocular pressure is elevated, and Perfluorocarbon (PFCL) is used to prevent or minimize bleeding.

4) The intraocular pressure is lowered to 15 to 20 mmHg, and a shallow retinal detachment is created by subretinal injection with Ringer's solution;

5) With the help of a forcep the prepared sheet of aminiotic membrane with RPE monolayer is delivered into the subretinal area in the foveal area;

6) Finally, an air or gas temponade is made to secure the transplant sheet in position; the sclerotomies are closed; and the patient is asked to remain in a prone position for the next few days.

It should be noted that, for cell harvesting only, a second retinotomy on the nasal side is performed.

In yet another aspect of the invention, an embodiment of a device for harvesting RPE cells was conceived and designed. The device, the Binder RPE harvester cannula, minimizes damage to the RPE and maximizes harvest yield. The device has several features and is described as follows:

The outer diameter of the harvester cannula is a standard 20ga (0.9mm) at the sclerotomy site to match current vitrectomy instruments. Its proximal end is terminated with a conventional LUER female connector fitting a 0.5cc glass syringe. Its distal end is bent (10mm radius of curvature). The last 5 mm of the distal tip, that will be placed in contact with the retinal tissues, has a flattened crescent-shaped hollowed cross-section with a radius of curvature matching the surface of Bruch's membrane at the posterior pole. That radius is 11mm approximately. The end of the tip has a 10 degree taper forming a lower lip and a upper lip. Therefore the lower lip protrudes forward and is visible at all times through the operation microscope (surgeon's view). Because of the gentle taper, the upper lip of the tip is far away from the lower lip, thus exposing the hollow in the cannula which also becomes visible to the surgeon. The thickness of each lip is of ~75 micrometers, the hollow bore is of 100 micrometers in height, making the thickness of the tip 250 micrometers. The width of the cannula's outer surface at its distal end is approximately 1.5mm, the width of the hollow bore is approximately 1.35mm. The lower lip taper is finished with a polished round edge of approximately 3 micrometers. As the lower lip is slightly tapered, and as the retinal tissue is very elastic, the retinotomy width needs to be only 1mm approximately.

The Binder RPE harvester cannula has a flattened crescent shape with a forward protruding lip that can glide on the surface of the elastic Bruchs membrane supporting the RPE cells. This will maximize RPE cell harvesting over a width of approximately 1.2mm per pass. As the posterior curvature of

the cannula's lip matches that of Bruch's membrane, it will minimize damage to the membrane itself (no cutting, stripping or pitting can occur). Because its cross-sectional shape is closest to the length and shape of the retinotomy opening, a good fit will be obtained between the cannula outer wall and the edges of the retinotomy. This will minimize trauma to the retina and prevent or minimize backflush of RPE cells into the vitreous cavity. The Binder RPE harvester cannula can be made of stainless steel or plastic materials.

EXEMPLIFICATION

Animals

Dutch belted rabbits were obtained from Covance Research Products, Inc. (Denver, PA, USA). All rabbits used were euthanised by intramuscular injection of 0.3 ml ketamine (35 mg/kg) and xylazine (5 mg/kg) followed by an injection of 1ml of Euthasol® (Delmarva Laboratories, Inc., Midlothian, VA).

Materials

Dulbecco's modified Eagle's medium with F12 nutrient mixture 1:1 (V/V), dialyzed fetal bovine serum (FBS) with molecular cut off rate of 10,000 daltons, L-glutamine, L-methionine, L-lysine, L-leucine, magnesium chloride, magnesium sulfate, calcium chloride, cell culture grade water, sodium bicarbonate, FITC-conjugated goat anti rabbit IgG, and mouse monoclonal anti-cytokeratin (CK) 18 (clone CY-90) were all obtained from Sigma-Aldrich Chemical Company (St. Louis, MO, USA). Phenol red sodium, DMEM/F12, sterile phosphate buffered saline (PBS), amphotericin B, trypan blue stain solution, and trypsin/EDTA were purchased from Gibco BRL (Grand Island, NY, USA).

Twenty-four well plates were used. (Corning Life Sciences) Collagenase type I with 239 U/mg was purchased from Worthington Biochemical Corporation (Lakewood,

NJ, USA). Penicillin and streptomycin were obtained from Bio Whittaker (Walkersville, MD, USA). Goat anti-rat Alexa 546- conjugated IgG (H+L), F(ab)2, Goat anti-mouse Alexa 488-conjugated IgG F(ab)2 were purchased from Molecular Probes Inc. (Eugene,OR, USA). Aqua-Poly/mount, was obtained from Polysciences Inc. (Warrington,PA, USA). The polyclonal rabbit anti-RPE-65 antibody was a generous gift from T. Michael Redmond.

The monoclonal rat anti-ZO-1 antibody (MAB 1520) was obtained from Chemicon (Temecula, CA, USA). The monoclonal mouse anti-Pancytokeratin K8.13 Ab was obtained from ICN Biomedicals, Inc. (Aurora, OH, USA). As previously published 13, for the epithelial denudement of the amniotic membrane a corneal epithelial scrubber was employed (Amoils Epithelial Scrubber; Innova, Innovative Excimer Solutions, Inc., Toronto, Ontario, Canada). Culture plate inserts used for fastening amniotic membrane were from Millipore (Bedford, MA, USA).

Primary RPE Cultures

Following euthanasia, eyes were immediately enucleated, and the anterior segment was removed by circumferential incision with scissors ca. 3-4 mm posterior to the corneal limbus. The isolation of RPE followed what has been reported 48 except that collagenase was used. In brief, the neural retina was detached by subretinal injection of sterile PBS, pH 7.4, to facilitate the removal of the vitreous residues and the neural retina. The RPE surface was then rinsed 3 times with PBS, and the eyecup was incubated with 1 mg/ml collagenase type I in DMEM/F12 for 1 h at 37 °C in the incubator with 5% CO_2. RPE sheets were collected by gentle shaving of the Bruch's membrane with a heat-polished glass pipette. The cells were centrifuged at 800 rpm for 5 min and plated into 24 well plates (5-6 wells per eye) in DMEM/F12 adjusted to 0.1 mM Ca^{++}, supplemented with 10% dialyzed FBS, 100 IU/ml

Penicilin, (100 g/ml streptomycin and 0.5 g/ml amphotericin B). The cultures were cleared of the debris 48 h after plating by changing 50% of the medium with a fresh medium. The medium was then completely changed biweekly.

Amniotic Membrane Preparation

Human amniotic membrane (AM) was kindly provided by Bio-Tissue, Inc. (Miami,FL) according to the method previously described (US Patents Nos. 6,152,142 and 6,326,019), and stored in DMEM and glycerol (1:1) at -80°C before use. Upon use, AM was thawed at room temperature and rinsed with sterile Hanks Balanced Salt Solution (HBSS) to remove excess glycerol, and sutured with 4-0 silk surgical sutures (Alcon Surgical, USA) and/or tightened by a rubber-ring with the epithelial side facing up to 24 well plate culture insert as previously described 63. In a separate experiment, AM was epithelially denuded by incubation with sterile 0.02% EDTA in PBS for 45min followed by gentle polishing with an epithelial scrubber (Amoils Epithelial Scrubber; Innova, Innovative Excimer Solutions, Inc., Toronto, Ontario, Canada) as previously described 62. After rinsing with sterile HBSS for 3 times, these AM were stored for 3 to 4 days in DMEM/ F12 before seeding with RPE cells.

Passage of RPE Cells

First passage of RPE cells were obtained by the treatment of 0.05 % trypsin and 0.02 % EDTA in Ca++ and Mg++-free HBSS for 8 min when they were in the late log phase, and seeded at 5,000-20,000 viable RPE cells per cm^2 (as assessed by trypan blue staining) in DMEM/F12 with Ca++ concentrations adjusted to 0.1 mM, and supplemented with 10% dialyzed FBS, 100 IU/ml PNS and 0.5 (μg/ml amphotericin B on either plastic, denuded AM or intact AM.

Cells were left undisturbed for 36 h to allow attachment, and culture media were changed biweekly thereafter.

Calcium Switch

When RPE cells on each of the above culture reached confluence, the Ca++ concentration in the culture medium was changed to 1.8 mM by adding soluble calcium salt.

Evaluation

Each culture was followed with observation under a phase contrast microscope at 36 h after plating, at confluence, and one week and four weeks after the calcium switch.

At different intervals, cultures were terminated by removing the medium, and rinsing the cells 3 times with sterile PBS. Thereafter, the cultures were fixed either in pre-cooled (-20°C) methanol for 5 min for Cytokeratins or in 4% paraformaldehyde at 4°C for 10 min (for RPE-65, ZO-1). This was followed again by rinsing 3 times in PBS. The tissue was thereafter stored at 4 °C in 0.01 NaN_3 in PBS for about 2 weeks until further processing. Primary antibodies were incubated overnight at 4 °C at the following concentrations: Pancytokeratin (K8.13) at 1/100, Cytokeratin 18 (CY-90) 1/3000 (both according to [25]), ZO-1 at 1/200 and the RPE-65 at 1/200. This was followed by 3 washes in PBS and then incubated for 2 h with respective secondary antibodies conjugated to either FITC (for RPE-65), Alexa 546 (for ZO-1), Alexa 488 (for Pancytokeratin and Cytokeratin 18) in a dilution of 1/200. All antibodies were diluted in PBS containing 1% BSA and 0.1% Triton x-100. The specimens were washed 3 times in PBS and mounted immediately in Aqua-Poly/mount mounting medium. The staining was then analyzed with a Zeiss

Axiophot fluorescence microscope (Zeiss, Oberkochen, Germany) which was connected to a CCD Optronics camera. The images were subsequently enhanced with Adobe Photoshop 5.5 software.

For immunostaining, the specificity of the above antibodies had been verified in dutch belted rabbit tissue. In brief, an animal, previously used for another procedure requiring subsequent euthanasia, was perfused under anaesthesia with 4% formaldehyde. Thereafter an injection of 4% paraformaldehyde in PBS was given intravitreally and the eye immersed in the same fixative and kept on ice for 2 h. The anterior segment was then dissected away and the vitreous removed as much as possible. Samples were cut with ophthalmic scissors from the posterior pole of the eye and the cornea , incubated in PBS with sequential sucrose gradients of 10, 20 and 30%, embedded in OCT and snap frozen with liquid nitrogen. Tissue sections were cut at 8 μm on a Reichert Cryostat.

RESULTS

Morphological Appearance of RPE Grown in Low Calcium on Different Substrates

Rabbit RPE cells were seeded at 5,000-20,000 viable RPE cells per cm^2 on plastic (P), epithelially denuded human amniotic membrane (dAM), or intact human amniotic membrane (iAM) in low Ca++ DMEM/ F12. Confluence was reached in about 7-9 days on dAM, which was faster than 9-10 days on plastic. As shown in Fig. 1, RPE cells on these three cultures in general appeared spindle-shaped and were spread evenly on both substrates except that RPE cells on iAM (Fig. 1C) appeared to be more squamous and polygonal and less evenly distributed when compared to the plastic culture (Fig. 1A) and dAM (Fig. 1B) when confluence was reached. Furthermore, pigmentation of

melanolipofuscin granules rapidly disappeared on plastic cultures (Fig. 1A) in part due to dilution by cell division, a phenomenon that has been well recognized 33. Nevertheless, RPE cells on dAM still retained some granular appearance although pigmentation was also reduced (Fig. 1B), whereas RPE cells on iAM still possessed heavy pigmentation (Fig. 1C). All Figure 1A, B, and C were taken at same magnification (10x), while Fig. 1D is a higher magnification (40x) of Fig. 1C.

Morphological Appearance of RPE One Week after Calcium Switch

When RPE cells reached confluence on dAM, the medium was switched to high Ca++ DMEM/F12. As shown in Fig. 2, one week after calcium switch, RPE cells on plastic cultures remained spindle-shaped, but did not form clear granules except some pigmentation might reappear (Fig. 2A). In contrast, RPE cells grown on dAM adopted epithelioid appearance with a polygonal (hexagonal) shape, and exhibited abundant granules with pigmentation in the cytoplasm (Fig. 2B). Both Fig. 2A and 2B are taken at a higher magnification (40x) RPE cells on iAM also adopted polygonal shape with heavy pigmentation (Fig. 1C). As a comparison, RPE cells grown on dAM and low calcium still retained spindle shape but appeared to have more pigmentation (Fig. 1D). Figure 1C and 1D are of the same low magnification (10x).

Characterization of Resultant Epithelial Phenotypes by Immunostaining

CK18 Staining

Figure 3 shows the staining by antibody to CK18 (cytokeratin 18), a marker to identify the epithelial origin of RPE cells. The results indicate that indeed RPE cultured on plastic under low Ca^{2+} had the epithelial origin because all cells were positive (Fig. 3A). RPE growing on intact (Fig. 3C) and denuded (Fig. 3D) showed strong positive

staining with vivid cytoskeleton pattern (Fig. 3D), which was more pronounced then counterpart growing on plastic when Ca was elevated (Fig. 3B).

RP 65 Staining

Figure 4

Figure 4 shows the staining to RP65, which a new marker for RP differentiation. Here shows the normal pattern of positive staining of RPE in vivo rabbit retina, showing a monolayer of RPE between the photoreceptors (Ph, on the top) and choriocapillaris (Ch, on the bottom), and was pigmented (marked by *)

RP 65 Staining

Figure 5

Figure 5: RP65 staining showed that RPE grown on plastic was negative even 1.5 weeks after Ca^{2+} switch (Fig. 5A). In contrast, RPE cells were strongly positive to RP65 when grown on intact AM (Fig.5B) and on denuded AM (Fig.5C). This result continued when the culture was extended to 3.5 weeks (Fig. 5D and 5E, respectively). The positive staining is shown by green fluorescence, while the reddish staining was nuclear counterstaining by PI.

ZO-1 Staining

Figure 6

ZO-1 staining is directed to the tight junction complex formed by RPE. *In vivo,* this antibody to ZO-1 showed positive (reddish fluorescence) in the RPE and photoreceptor complex (indicated by a bracket).

ZO-1 Staining

Figure 7

ZO-1 staining was also positive on the RPE cells grown on plastic (Fig. 7A, B) and intact amniotic membrane (Fig. C1, 2) and denuded AM (Fig. D1, 2).

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EQUIVALENTS

While this invention has been particularly shown and described with references to preferred embodiments thereof, it will be understood by those skilled in the art that various changes in form and details may be made therein without departing from the scope of the invention. Those skilled in the art will recognize, or be able to ascertain using no more than routine experimentation, many equivalents to the specific embodiments described herein. Such equivalents are intended to be encompassed in the scope of the following claims.

CLAIMS

What is claimed is:

1. A composite for implantation in the subretinal space of an eye of a patient in need thereof, comprising:

a) amniotic membrane; and

b) a plurality of retinal pigment epithelial cells or retinal pigment epithelial equivalent cells present at the amniotic membrane.

2. The composite of Claim 1, wherein the amniotic membrane is epithelially denuded.

3. The composite of Claim 1, wherein the amniotic membrane is intact amniotic membrane comprising a basement membrane and a stroma.

4. The composite of Claim 3, wherein the amniotic membrane is present as a membrane treated on at least one side.

5. The composite of Claim 4, wherein the treatment is excimer laser ablation to thin the stromal side or excimer laser ablation to thin the basement membrane side.

6. The composite of Claim 4, wherein the treatment is laser treatment to alter the thickness of the stromal side.

7. The composite of Claim 4, wherein the treatment is addition of mesenchymal cells to the stromal side.

8. The composite of Claim 7, wherein the cells are fibroblasts.

9. The composite of Claim 1, where in the amniotic membrane is human amniotic membrane.

10. The composite of Claim 1, wherein the number of retinal pigment epithelial equivalent cells at the amniotic membrane is between about 16,000 and about 20,000 per $4mm^2$.

11. The composite of Claim 1, wherein the number of retinal pigment epithelial equivalent cells at the amniotic membrane is about 4,000 per mm^2.

12. The composite of Claim 1, wherein the retinal pigment epithelial equivalent cells comprise iris pigment epithelial cells.

13. The composite of Claim 1, wherein a source of the retinal pigment epithelial equivalent cells comprises cells that have been immortalized by viral agents or non-viral agents.

14. The composite of Claim 1, wherein a source of the retinal pigment epithelial equivalent cells comprises at least one stem cell induced *in vitro* to differentiate into a retinal pigment epithelial cell.

15. The composite of Claim 14, wherein the stem cells comprise adult stem cells.

16. The composite of Claim 14, wherein the stem cells comprise embryonal stem cells.

17. The composite of Claim 15, wherein the adult stem cells comprise peripheral blood cells or bone marrow cells.

18. The composite of Claim 1, wherein a source of the retinal pigment epithelial equivalent cells comprises at least one bioengineered cell induced *in vitro* to differentiate into a retinal pigment epithelial cell.

19. The composite of Claim 1, wherein the retinal pigment epithelial equivalent cells retain the retinal pigment epithelial phenotype.

20. The composite of Claim 1, wherein the retinal pigment epithelial equivalent cells present at the amniotic membrane comprise cultured cells.

21. The composite of Claim 1, wherein the retinal pigment epithelial equivalent cells comprise cells derived from neural retinal cells.

22. The composite of Claim 21, wherein the neural retinal cells comprise rod cells or cone cells.

23. The composite of Claim 1, further including a pharmaceutically active molecule.

24. The composite of Claim 23, wherein the pharmaceutically active molecule comprises at least one substance independently selected from the group consisting of growth factors, enzymes, and therapeutic drugs.

25. The composite of Claim 24, wherein the growth factor is selected from the group consisting of retinal pigment epithelium-derived growth factor and transforming growth factor-beta.

26. The composite of Claim 23, wherein the pharmaceutically active molecule is interleukin-10.

27. A kit comprising:

a) amniotic membrane;

b) a plurality of retinal pigment epithelial cells or retinal pigment epithelial equivalent cells present at the amniotic membrane;

c) a buffer medium or a culture medium; and

d) optionally, instructions for simultaneous, separate, or sequential use of at least one component of the kit for treating a retinal disease.

28. The kit according to Claim 27, further comprising at least one pharmaceutically active agent.

29. The kit according to Claim 28, wherein the pharmaceutically active agent comprises at least one substance independently selected from the group consisting of growth factors, enzymes, and therapeutic drugs.

30. The kit of Claim 29, wherein the growth factor is selected from the group consisting of retinal pigment epithelium-derived growth factor and transforming growth factor-beta.

31. The kit of Claim 28, wherein the pharmaceutically active agent is interleukin-10.

32. The kit according to Claim 27, wherein the retinal pigment epithelial equivalent cells comprise bioengineered cells.

33. A method of forming a composite for implantation in the subretinal space of an eye of a host in need thereof, comprising the steps of:

a) applying at least one retinal pigment epithelial cell or retinal pigment epithelial equivalent cell to an amniotic membrane; and

b) culturing the retinal pigment epithelial cell or retinal pigment epithelial equivalent cell on said membrane under conditions suitable for growth for a period of time sufficient to produce a plurality of cultured cells.

34. The method of Claim 33, wherein the number of cells cultured on said membrane is between about 16,000 and about 20,000 per $4mm^2$.

35. A method of inducing an excised or cultured retinal pigment epithelial cell or retinal pigment epithelial equivalent cell to express or to maintain the phenotype of retinal pigment epithelial cells, the method comprising the steps of:

a) contacting amniotic membrane with the retinal pigment epithelial cell or retinal pigment epithelial equivalent cell;

b) culturing the retinal pigment epithelial cell or retinal pigment epithelial equivalent cell on said membrane under conditions suitable for growth for a period of time sufficient to produce a plurality of cultured cells; and either

c) contacting the cultured cells with an effective amount of an agent that raises the intracellular calcium ion concentration to a level sufficient to induce or maintain the phenotype of retinal pigment epithelial cells; or

d) exposing said membrane comprising cultured cells to an air-fluid interface for a period of time sufficient to induce or maintain the phenotype of retinal pigment epithelial cells.

36. The method of Claim 35, wherein both steps c and d are performed.

37. The method of Claim 35, wherein in step c, the intracellular calcium ion concentration is elevated to a range of about 0.5 mM to about 2.0 mM;

38. The method of Claim 35, wherein in step c, the intracellular calcium ion concentration is elevated to about 1.8 mM.

39. The method of Claim 35, wherein the step of culturing the retinal pigment epithelial cell or retinal pigment epithelial equivalent cell on said membrane is continued until the cells reach confluence.

40. The use of human amniotic membrane to promote growth and differentiation of at least one retinal pigment epithelial equivalent cell to a plurality of cells that express the phenotype of retinal pigment epithelial cells.

41. A method of delivering a plurality of retinal pigment epithelial cells to a target site in a subretinal space in an individual in need thereof, comprising:

a) forming at least one hole in a retina of the individual, or at least partially detaching the retina to access the subretinal space;

b) inserting through the hole a composite comprising amniotic membrane and the retinal pigment epithelial cells present at the membrane; and

c) positioning said composite at the target site.

42. A method for treating a retinal disease, comprising inserting in a subretinal space of a patient in need thereof a composite comprising amniotic membrane and a plurality of retinal pigment epithelial cells present at said membrane.

43. The method of Claim 42, wherein the number of retinal pigment epithelial cells present at said membrane is between about 16,000 and about 20,000 per $4mm^2$.

44. The method of Claim 42, wherein the retinal disease that is treated is age-related macular degeneration.

45. The method of Claim 42, wherein the retinal disease that is treated is selected from the group consisting of retinal detachment, gyrate atrophy, and choroideremia.

46. The method of Claim 42, wherein the amniotic membrane is human amniotic membrane.

47. The method of Claim 42, wherein the retinal pigment epithelial cells comprise cells cultured on the amniotic membrane.

48. The method of Claim 42, wherein the composite further comprises a pharmaceutically active molecule.

49. The method of Claim 48, wherein the pharmaceutically active molecule is selected from the group consisting of growth factors, enzymes, and therapeutic drugs.

50. The use of human amniotic membrane in transplanting retinal pigment epithelial cells or iris pigment epithelial cells to a subretinal space, to prevent or decrease a sensitizing of a recipient to alloantigens and to retinal pigment epithelial specific autoantigens.

51. The use of human amniotic membrane to inhibit fibrosis following transplantation of retinal pigment epithelial cells or iris pigment epithelial cells to the subretinal space.

52. The use of human amniotic membrane and at least one retinal pigment epithelial cell for the manufacture of a composition for treatment of a retinal disease in a patient suffering from, or at risk of developing the disease.

RETINAL PIGMENT EPITHELIAL CELL CULTURES ON AMNIOTIC MEMBRANE AND TRANSPLANTATION

ABSTRACT OF THE DISCLOSURE

The present invention relates to a composition for implantation in the subretinal space of an eye, the composition including amniotic membrane, which may be cryopreserved human amniotic membrane, and a plurality of retinal pigment epithelial (RPE) cells or RPE equivalent cells present at the amniotic membrane. The amniotic membrane may be intact, epithelially denuded, or otherwise treated. The invention includes the use of amniotic membrane for the culturing of RPE cells thereon, forming a surgical graft for replacement of RPE, Bruch's membrane, and underlying choriocapillaris. The composite does not elicit immunological reactions to alloantigens or to RPE specific autoantigens; and exerts anti-inflammatory, anti-angiogenic, and anti-scarring effects. The invention includes methods and kits for making or using composites including amniotic membrane and RPE cells.

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Applicants: Binder, et al. Atty. Dkt. No.: 2119.003P

Serial No.: UNKNOWN

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Title: RETINAL PIGMENT EPITHELIAL CELL CULTURES ON AMNIOTIC MEMBRANE AND TRANSPLANTATION

Box Patent Application
Assistant Commissioner for Patents
Washington, D.C. 20231

ASSERTION OF SMALL ENTITY STATUS UNDER 37 CFR §1.27(c)(2)

Dear Sir:

The Assignee of the above-identified application, TissueTech, Inc., qualifies for Small Entity Status. Thus, the filing fees submitted herewith are for a Small Entity.

Respectfully submitted,

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Fig. 1

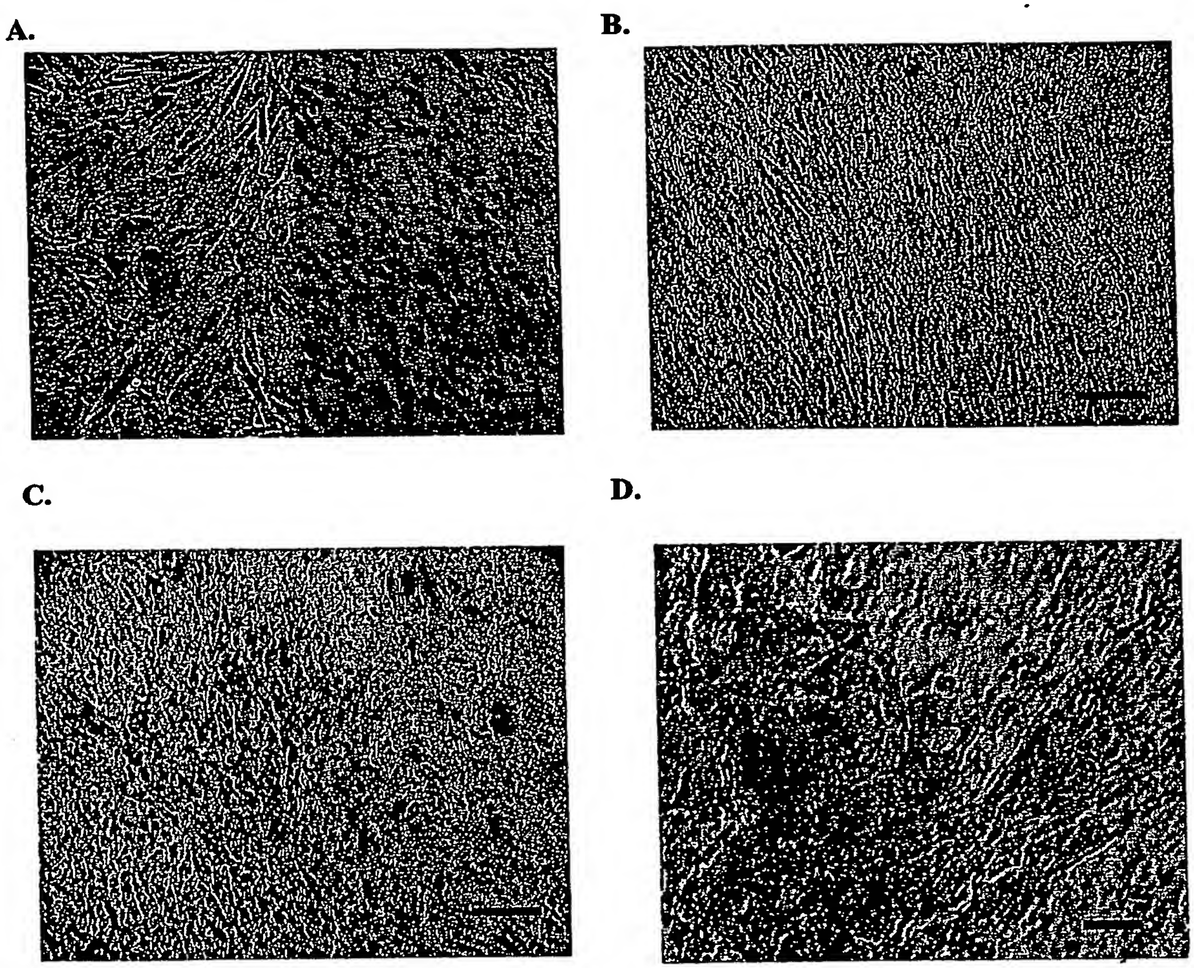


Fig. 2

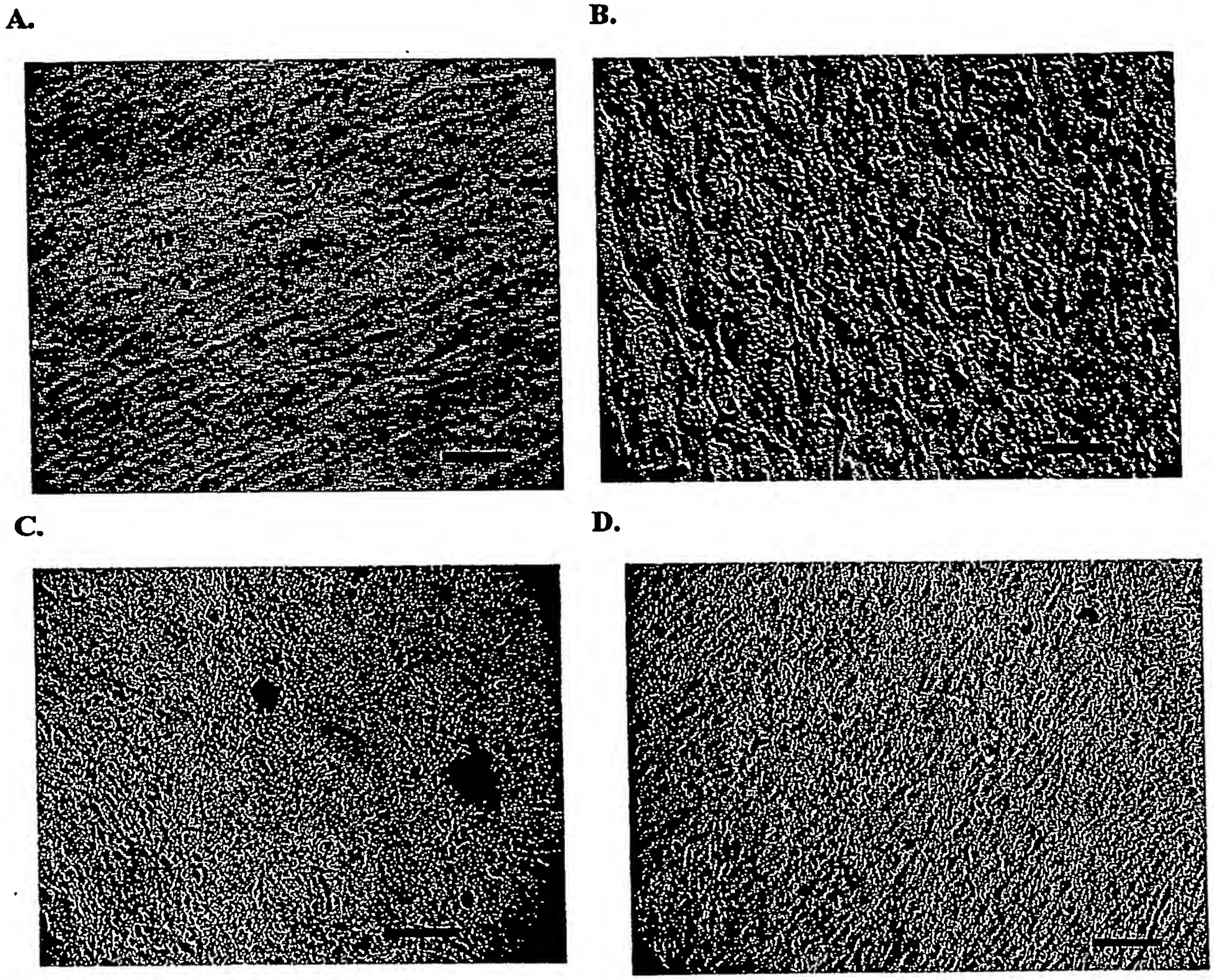


Figure 3

A. B.

C. D.

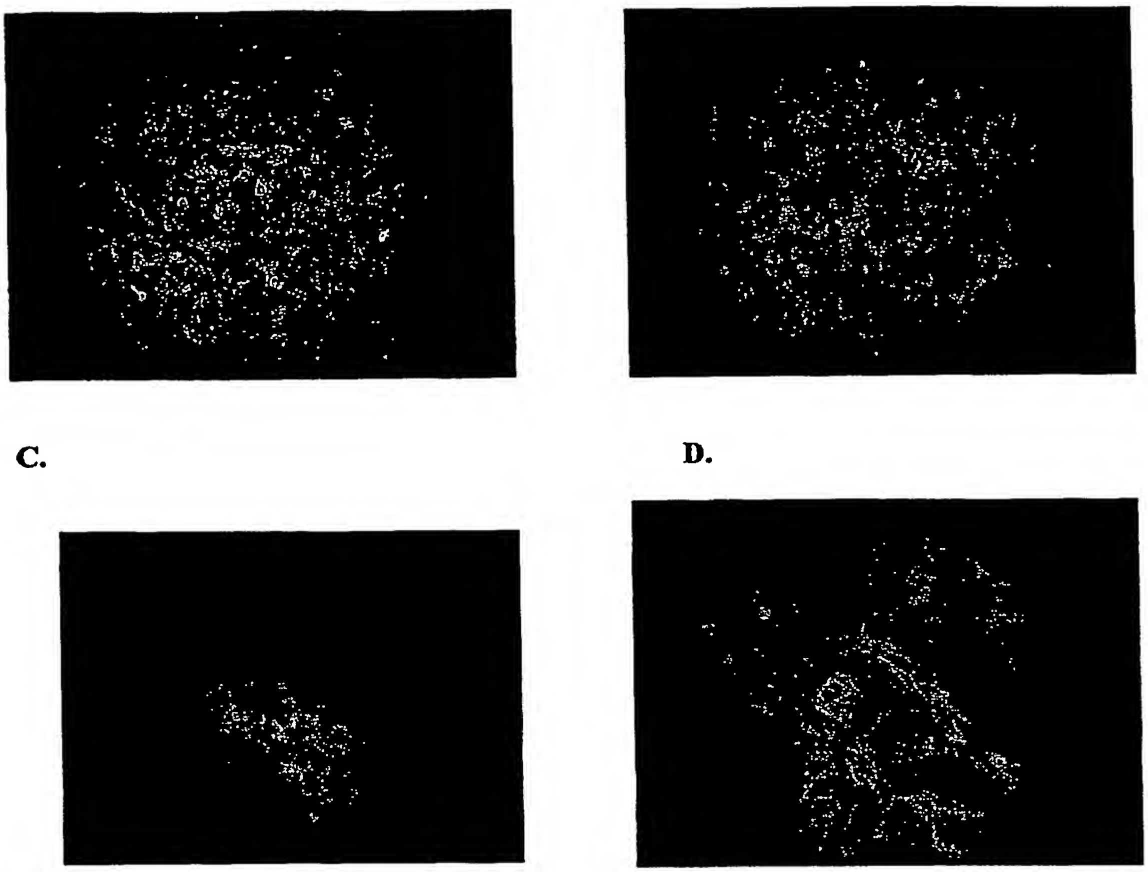
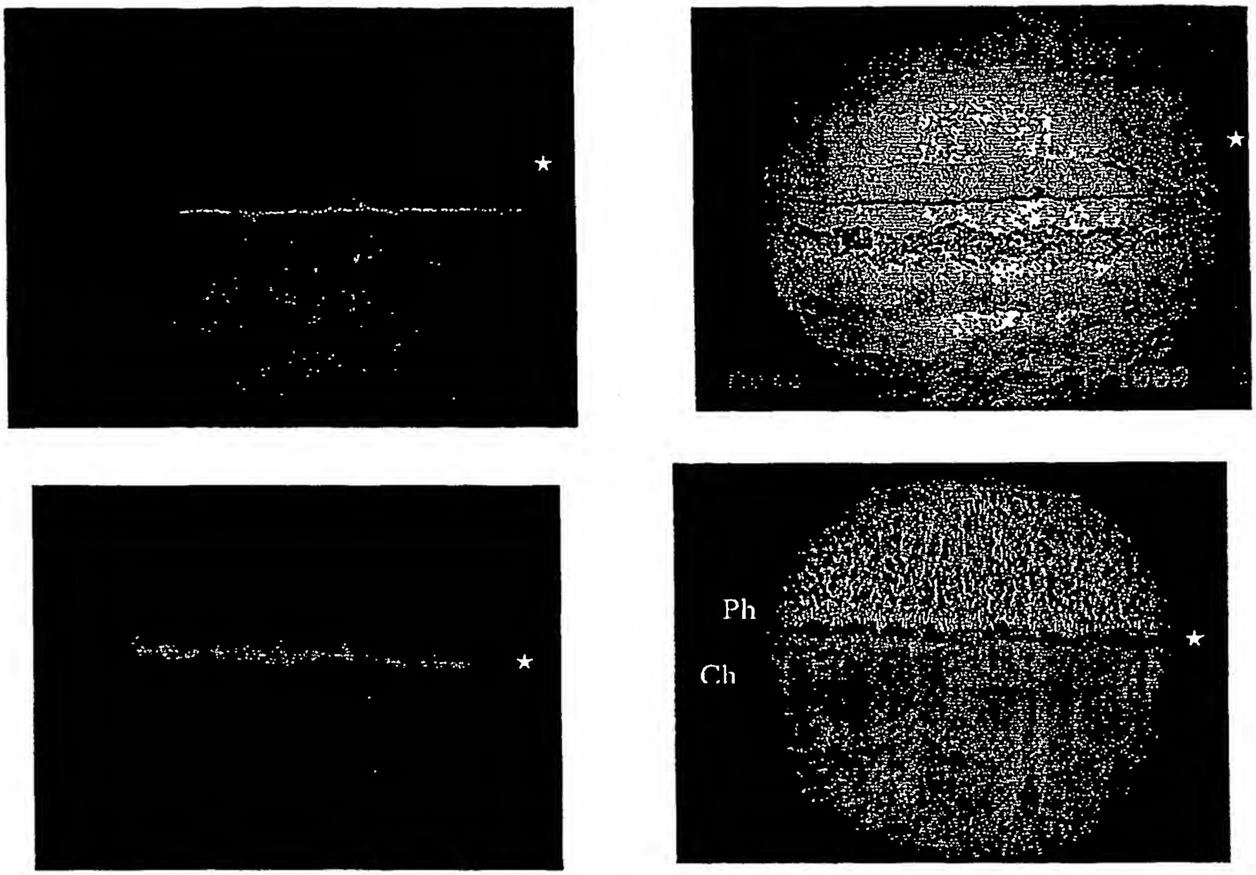


Figure 4

A. B.



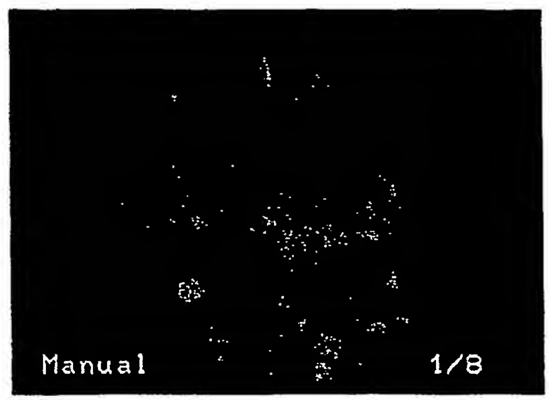
C. D.

Figure 5

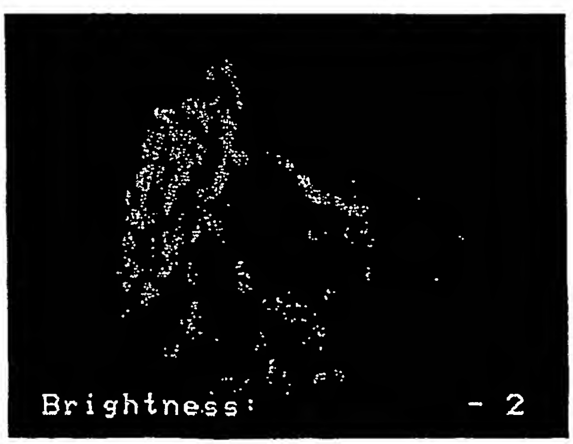
A.

B.

C.



D.



E.

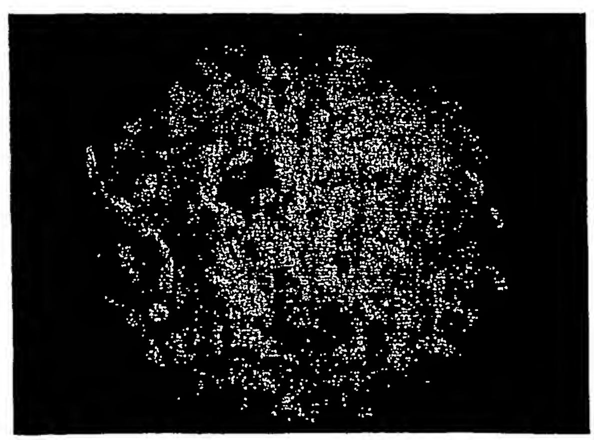
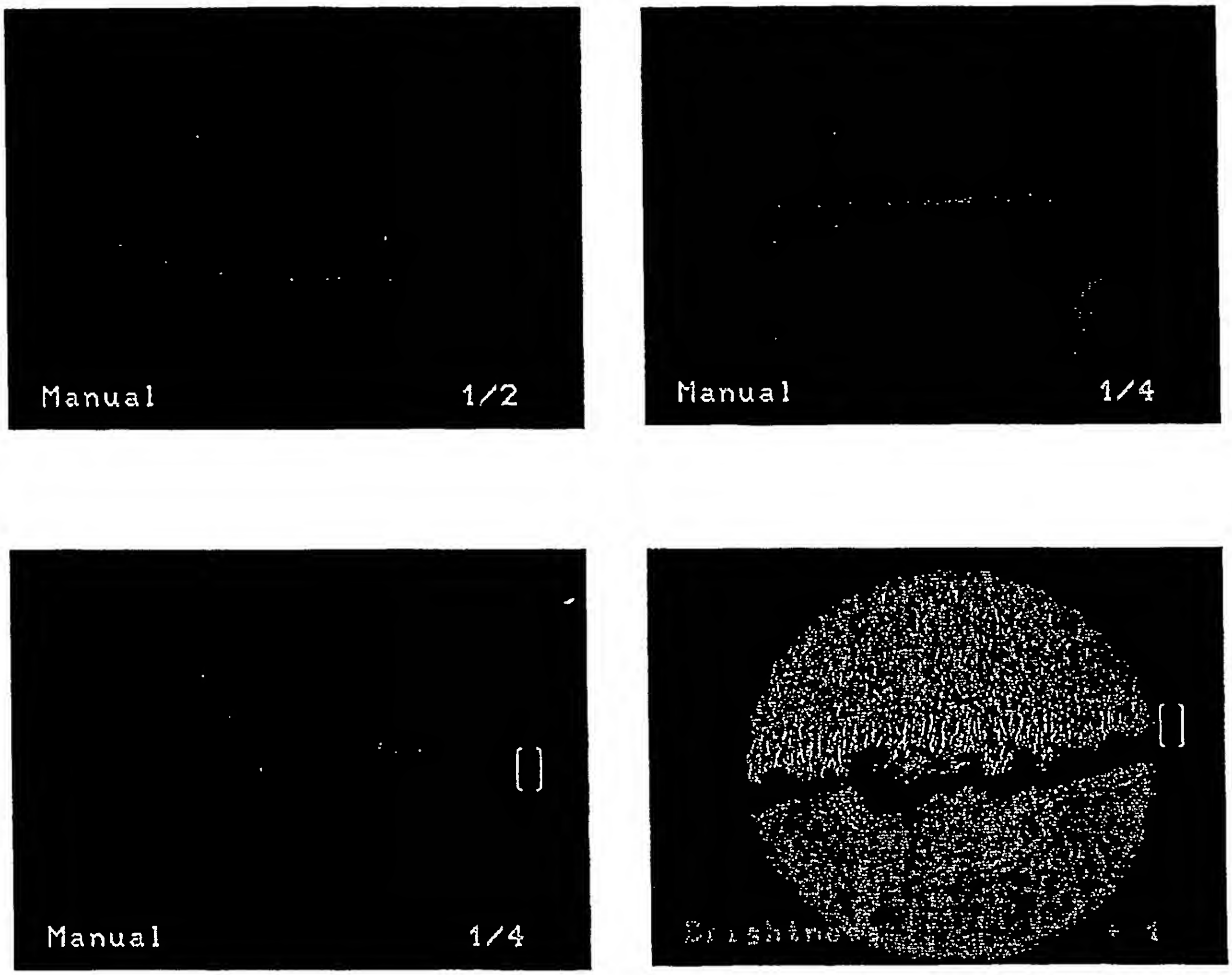


Figure 6

A. **B.**

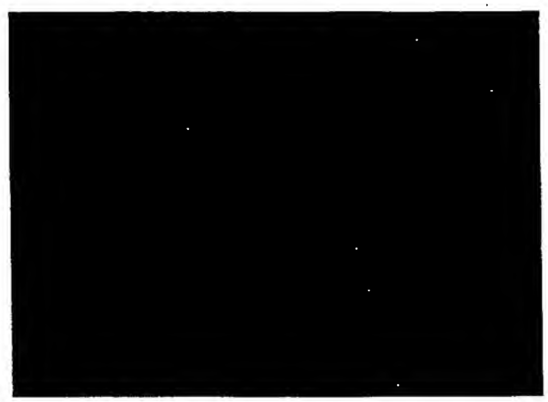


C. **D.**

Figure 7

A. 1

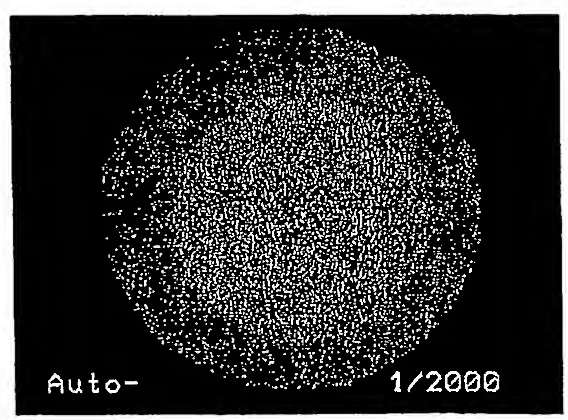
A. 2



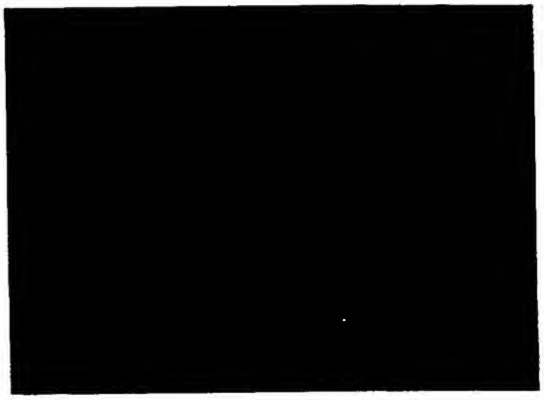
B.

C. 1

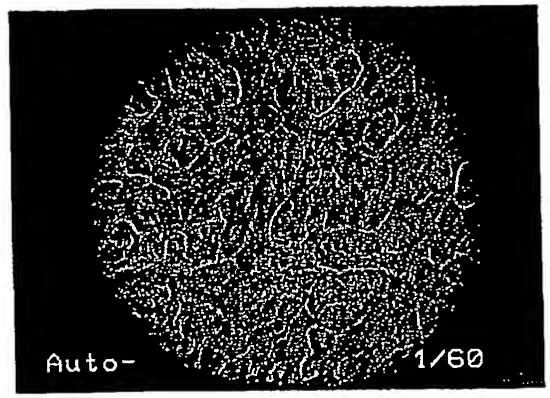
C. 2



D. 1



D. 2



Connexin 43 Expression and Proliferation of Human Limbal Epithelium on Intact and Denuded Amniotic Membrane

Martin Grueterich,[1] *Edgar Espana,*[1] *and Scheffer C. G. Tseng*[1,2]

Purpose. Stem cell (SC)–containing limbal basal epithelium and transient amplifying cell (TAC)–containing corneal basal epithelium lie on different mesenchymal matrices. The gap junction protein connexin 43 (Cx43) is absent in the limbal basal epithelium but is present in the corneal basal epithelium, suggesting that the expression of Cx43 denotes SC differentiation into TACs. Amniotic membrane (AM) can expand limbal epithelial progenitor cells in vivo and in culture for subsequent corneal surface reconstruction. In this study, the modulation of Cx43 expression, gap junction intercellular communication (GJIC), and proliferative activity of ex vivo expanded human limbal epithelial (HLE) cells on intact and epithelially denuded AM was investigated.

Methods. HLE cells were expanded on intact (i.e., remaining devitalized amniotic epithelium) or epithelially denuded AM (EDTA-treated). Cx43 expression and 24-hour 5-bromo-2′-deoxyuridine-5′monophosphate (BrdU) labeling index (percentage) were determined by double immunostaining. GJIC was investigated by a scrape-loading dye transfer assay. In a subset of cultures Cx43 and K3 keratin as well as BrdU-retaining nuclei were analyzed in the stratified epithelium obtained 5 days after subcutaneous transplantation in NIH bg-nu-xidBR mice of AM cultures continuously labeled with BrdU for 7 days.

Results. The outgrowth rate, overall, was significantly higher on EDTA-treated AM than on intact AM ($P < 0.05$). Cx43 was expressed in 12.4% ± 14.5% ($n = 5$) on intact and 57.5% ± 18.2% ($n = 5$) on EDTA-treated AM ($P < 0.05$). The BrdU labeling index was 2.4% ± 0.9% ($n = 5$) for the intact AM group, which was significantly less than 22.5% ± 8.2% ($n = 5$) for EDTA-treated AM ($P < 0.05$). BrdU-labeled cells did not express Cx43. The dye transfer assay revealed reduced GJIC on both AM-cultured groups compared with the control culture on plastic ($P < 0.002$). GJIC on intact AM (17%) was reduced compared with that on EDTA-treated AM (27%; $P = 0.42$). After xenotransplantation, the basal layer of the stratified epithelium was Cx43 and K3 keratin negative and retained BrdU on intact AM, resembling characteristics of the limbal basal epithelium in vivo. In contrast, that of EDTA-treated AM was Cx43 and K3 keratin positive without BrdU retention, resembling characteristics of the corneal epithelium in vivo.

Conclusion. These data indicate that denudation of the devitalized amniotic epithelium to expose its basement membrane might be a microenvironmental cue to promote TAC differentiation. The model system described herein is ideal for future exploration of the exact mechanistic operation in the microenvironmental niche that maintains the "stemness" of limbal SCs as well as in the signal that promotes corneal TAC differentiation. (*Invest Ophthalmol Vis Sci.* 2002;43:63–71)

Stem cells (SCs) and transient amplifying cells (TACs) of the corneal epithelial progenitor population are located in the limbal and corneal basal layer, respectively.[1] The limbal epithelium lies on top of a loose and well-vascularized stroma, whereas the corneal epithelium lies on the compact and avascular Bowman's layer (i.e., a thick basement membrane). Phenotypic studies have shown that the SC-containing limbal basal epithelium does not express the cornea-specific K3/K12 keratin pair.[1–4] Cell cycle studies have shown that some limbal basal epithelial cells are slow cycling[5,6] and label retaining.[5,7] Of particular interest, expression of connexin 43 (Cx43) is noted in the human corneal but not limbal basal epithelial cell layer,[8–10] suggesting that the expression of Cx43 denotes differentiation of corneal TAC. Wolosin et al.[10] proposed that the absence of Cx expression may endow limbal epithelial SCs with the property of "stemness" in this microenvironmental niche, so that they can be segregated from differentiated TACs. It remains to be determined how the pool of limbal epithelial SCs is maintained to meet the conflicting demand of SC renewal and SC differentiation into TACs in this microenvironmental niche.

Clinically, limbal epithelial cells expanded in amniotic membrane (AM) cultures can restore the corneal surface with partial and total limbal SC deficiency.[11–13] Experimentally, AM has been shown to be an ideal substrate that preferentially expands the outgrowth of limbal biopsy specimens.[14,15] Such ex vivo expanded limbal epithelial cells are devoid of K3/K12 expression and remain slow cycling (Ref. 14 and manuscript submitted). Recently, we further noted that such ex vivo expanded limbal epithelial cells show no Cx43 expression and gap junction–mediated intercellular communication (GJIC; Ref. 16 and manuscript submitted). It should be noted that AM used in these studies was intact—that is, it retained a monolayer of devitalized amniotic epithelial cells. Koizumi et al.[15] recently reported that rabbit corneal and limbal epithelial cells grow much faster when cultured on epithelially denuded AM compared with those on intact AM with a layer of devitalized amniotic epithelium. Their morphologic results led to the conclusion that denuded AM with the exposed basement membrane is more suitable to support corneal epithelial cell growth for further transplantation.

In this study we investigated the differences in proliferative activity, Cx43 expression and GJIC of human limbal epithelial (HLE) cells expanded as a monolayer on cryopreserved, intact and epithelially denuded AM. Our data suggest that intact AM

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(i.e., retaining devitalized AM epithelium) is a more favorable microenvironment for the expansion of SC-containing limbal epithelium than is EDTA-denuded AM (i.e., exposed AM basement membrane) with respect to the preservation of a Cx43-negative and GJIC-deficient phenotype. This notion is further supported by phenotypic characteristics of the expanded cell population after the induction of stratification through xenotransplantation into nude mice. The potential clinical significance of these two culture conditions for limbal epithelial cell expansion is further discussed.

Materials and Methods

Materials

Dulbecco's modified Eagle's medium (DMEM), HEPES buffer, amphotericin B, and fetal bovine serum (FBS) were purchased from Gibco BRL (Grand Island, NY). The mouse monoclonal IgG antibody against 5-bromo-2'-deoxyuridine-5'monophosphate (BrdU) and Dispase II were obtained from Roche Molecular Biochemicals (Indianapolis, IN). The FITC-conjugated goat anti-mouse IgG and IgM antibodies adsorbed with human serum proteins, gentamicin, hydrocortisone, dimethyl sulfoxide, cholera-toxin, insulin-transferrin-sodium selenite media supplement, lucifer yellow, rhodamine-dextran, EDTA, diaminobenzidine (DAB), propidium iodide, and Triton X-100 were all from Sigma Chemical Co. (St. Louis, MO). An ABC kit (Vectastain Elite) for mouse and rabbit IgG and the mounting medium (Vectashield) were obtained from Vector Laboratories (Burlingame, CA). The mouse monoclonal anti-Cx43 antibody was from Chemicon (Temecula, CA), and the polyclonal antibody against Cx43 was from Zymed (South San Francisco, CA). The IgG monoclonal antibody AE5, recognizing the 64-kDa keratin K3 was purchased from ICN (Costa Mesa, CA). The tissue culture plastic plates (six-well) were from Becton Dickinson (Lincoln Park, NJ). Culture plate inserts used for fastening AM were from Millipore (Bedford, MA).

Human Tissue Preparation

Human tissue was handled according to the Declaration of Helsinki. Corneoscleral tissues from human donor eyes were obtained from the Florida Lions Eye Bank (Miami, FL) immediately after the central corneal button had been used for corneal transplantation. The tissue was rinsed three times with DMEM containing 50 μg/mL gentamicin and 1.25 μg/mL amphotericin B. After careful removal of excessive sclera, iris, and corneal endothelium, the remaining tissue was placed in a culture dish and exposed to Dispase II (1.2 U/mL in Mg^{2+}- and Ca^{2+}-free Hanks' balanced salt solution) at 37°C under humidified 5% CO_2 for 5 to 10 minutes. After one rinse with DMEM containing 10% FBS, the scleral rim was trimmed to obtain limbal tissue cubes of approximately 1 × 1.5 × 2.5-mm size.

Human Limbus Cultures on Amniotic Membrane

Preserved human AM was kindly provided by Bio-Tissue (Miami, FL). AM was preserved according to the method described by Lee and Tseng.[17] Briefly, AMs derived from cesarean section placentas were rinsed in PBS containing 100 U/mL penicillin with 0.2 mg/mL streptomycin and stored in a solution of 50% DMEM and 50% glycerol at −80°C for at least 3 months. With this preservation method, both amniotic epithelium and stromal mesenchymal cells lose their viability and proliferative capacity.[18] After thawing at room temperature, AM with the epithelial side facing up was fastened onto a culture insert, as previously reported.[19] Fifty percent of the membranes used for limbal cultures were treated with 0.1% sterile EDTA solution for 30 minutes and then gently scrubbed with an epithelial scrubber (Amoils Epithelial Scrubber; Innova, Innovative Excimer Solutions, Inc., Toronto, Ontario, Canada), to remove the amniotic epithelium without breaking the underlying basement membrane. With this method, 90% to 100% of the epithelium could be removed. On the center of the AM, an explant was placed and cultured in a medium made of an equal volume of HEPES-buffered DMEM containing bicarbonate and Ham's F12. The medium was supplemented with 0.5% dimethyl sulfoxide, 2 ng/mL mouse epidermal growth factor (EGF), 5 μg/mL insulin, 5 μg/mL transferrin, 5 ng/mL selenium, 0.5 μg/mL hydrocortisone, 30 ng/mL cholera toxin A subunit, 5% FBS, 50 μg/mL gentamicin, and 1.25 μg/mL amphotericin B. Cultures were incubated at 37°C under 5% CO_2 and 95% air, and the medium was changed every 2 to 3 days. Each time the medium was changed, the outgrowth area was measured. When HLE cultures had almost reached confluent growth, defined as 270° of the circular outgrowth reaching the plastic culture ring or no further growth noticed after 4 weeks of culture, they were subjected to a semiquantitative dye transfer assay or incubated with 10 μM BrdU for 24 hours followed by fixation in cold methanol for immunostaining.

Xenotransplantation

All procedures were performed according to the ARVO Statement for the use of Animals in Ophthalmic and Vision Research. NIH bg-nu-xidBR mice, which have no thymus-derived T-cells, T-independent B lymphocytes, and natural killer cells, aged 6 to 10 weeks were purchased from Charles River Laboratories (Wilmington, MA). The animals were housed under temperature-, humidity-, and light- (12-hour light cycle; lights on at 7:00 AM) controlled conditions in filter-covered cages in a laminar flow–equipped room and kept on standard chow and water ad libitum. Before surgery, animals were anesthetized with intramuscular injection of 0.1 mL ketamine (35 mg/kg) and xylazine (5 mg/kg). Nearly confluent HLE cultures on intact and EDTA-treated AM were labeled with BrdU for 7 days to label rapid and slow-cycling cells and transplanted to the subcutaneous plane of the abdomen of NIH bg-nu-xidBR mice for a chase of 5 days, during which time only slow-cycling cells will retain the label. The mice were killed by craniocervical dislocation after intramuscular injection of 0.3 mL ketamine (35 mg/kg) and xylazine (5 mg/kg). The tissue, including implanted AM, was removed and embedded in optimal cutting temperature (OCT) compound for cryosectioning. Twelve cultures (six per condition) were transplanted.

Immunostaining

Frozen sections of 3 μm obtained from specimens after xenotransplantation were fixed in cold methanol for 20 minutes at −20°C followed by a 10-minute incubation in 0.1% Triton X-100 in PBS. After three rinses with PBS for 7 minutes each and preincubation with 5% BSA to block nonspecific staining, sections were incubated with a rabbit polyclonal anti-Cx43 (1:200), AE-5 (mouse anti-K3; 1:100), or mouse anti-BrdU (1:1000) monoclonal antibody for 45 minutes. After three washes with PBS for 15 minutes, the sections were incubated with an FITC-conjugated secondary antibody (goat anti-rabbit or anti-mouse IgG at 1:200) for 45 minutes. After three additional PBS washes (15 minutes each), they were mounted with an anti-fade solution (Vectashield, Vector Laboratories, Burlingame, CA) and analyzed with a fluorescence microscope (Axiophot; Carl Zeiss, Oberkochen, Germany).

For BrdU and Cx43 double-labeling, confluent cultures were incubated with 10 μM BrdU in the same culture medium for 24 hours. These cultures on AM were prepared as flatmount samples. After samples were air dried, rehydrated in PBS for 5 minutes, treated with 2 N HCl at 37°C for 45 minutes to denature DNA and neutralized in boric acid (pH 8.5) for 20 minutes, incorporated BrdU and Cx43 expression were detected by immunostaining with a mouse anti-BrdU antibody (1:1000) and a mouse anti-Cx43 antibody (1:200) followed by an ABC kit (Vectastain Elite) protocol (DAB-peroxidase staining). Samples were counterstained with hematoxylin. Under magnification of ×400, positive nuclei were counted among the total nuclei within the entire field, and a total of 16 fields were counted per specimen. The labeling index for BrdU was expressed as the number of positively labeled nuclei/the number of all nuclei × 100%. If all cells in one ×400 field expressed Cx43, we defined it as 1 unit of Cx43 expression. That is, if 50% of cells expressed Cx43, 0.5 unit was defined. We counted

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100 fields per sample for a total of 10 samples (n = 5 per condition) and reported their mean ± SD. Counterstaining on nude mice specimens to analyze BrdU retaining cells was performed with propidium iodide.

Semiquantitative Dye Transfer Assay

We used the scrape-loading dye transfer assay originally described by El-Fouly et al.[20] and Trosko et al.[21] For a positive control, we cultured HLE cells from an explant on plastic dishes for 14 days. HLE cells on plastic or AM were rinsed with sterile PBS. One milliliter lucifer yellow plus rhodamine-dextran (0.5 mg/mL) in PBS was added to the culture dish. A sterile scalpel blade was applied with gentle pressure to cut the cells. Six scrape lines were placed in different areas per culture. Dishes were left in a dark room for 3 minutes. Cells were rinsed extensively with PBS to prevent high background fluorescence. Cultures were fixed in 4% formalin and epifluorescence was examined using a fluorescence microscope (Axiophot; Zeiss) equipped with a UV light source. A rhodamine filter set was used to identify the red color of the primary loaded cells along the scrape line (absorbency 555 nm, emission 580 nm). Fluorescence filter sets were used to detect green fluorescence of lucifer yellow, which was transferred through gap junctions (absorbency 428 nm, emission 536 nm). We analyzed a total number of 54 scrape lines (6 scrape lines per culture for three separate cultures per condition; intact AM, EDTA-AM, control). The percentages of the entire length of all six scrape lines per culture were measured when we observed dye transfer more than four cell rows away from the initially loaded cells.

Statistical Analysis

A Greenhouse-Geisser corrected test of interaction between groups and the linear time component was used to evaluate the statistical significance between intact and EDTA-treated AM groups. The Mann-Whitney test was applied to compare the means of the cell density in both conditions. Data from the proliferation as well as from the Cx43 expression assay was analyzed by an unpooled variance approximate t-test. An ANOVA was applied to analyze the dye transfer assay. The Fisher exact test was used to compare samples with and without label-retaining basal cells after xenotransplantation. $P < 0.05$ was considered statistically significant.

Results

General Morphologic Features

Figure 1A shows a typical circular cell outgrowth of HLE cells on intact AM. The explant was surrounded by a cell layer termed the major outgrowth area. The leading edge of the outgrowth showed stratified cells composed of migrating HLE cells and devitalized amniotic epithelial cells. The leading edge was much smoother in the EDTA-treated group from which the amniotic epithelium had been removed before culture (Fig. 1B). Phase-contrast microscopy showed that the cell morphology of expanded HLE was small, compact, and uniform on both intact (Fig. 1C) and epithelially denuded AM (Fig. 1D), with the latter being more uniform than the former. The cell density was 2460 ± 220.1 cells/mm^2 on intact and 2820 ± 110 cells/mm^2 on EDTA-treated AM (P = 0.19, Mann-Whitney test). In most of the area of intact AM, we could identify expanding HLE cells on top of a layer of amniotic epithelial cells (Figs. 1E, 4E), whereas all HLE cells had direct contact with the amniotic basement membrane in EDTA-treated AM (Fig. 1F). The amniotic epithelium could be easily distinguished from overlying HLE cells, because the latter had a larger cell nucleus and cytoplasm.

Outgrowth Rate

The outgrowth area of 10 HLE cultures was photographed and measured each time the culture medium was changed, until

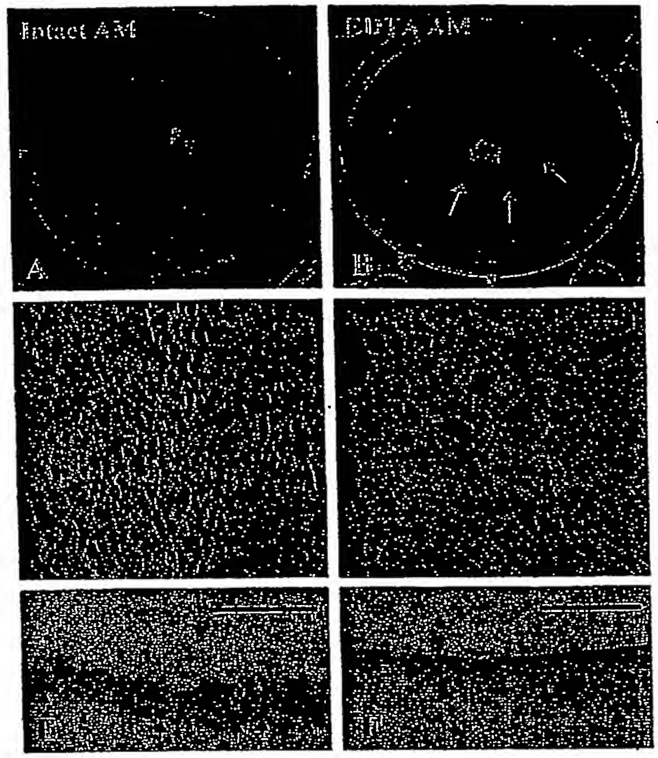

Figure 1. Morphology of HLE outgrowth on intact AM (*left column*) and EDTA-treated AM (*right column*). The outgrowth appearance from a biopsy explant on intact (A) and EDTA-treated AM (B) after 3 weeks and 1 week, respectively. Note the prominent leading edge on intact AM (*arrows*, A) compared with the smooth, discernible one on EDTA-treated AM (*arrows*, B). Phase-contrast microscopy of the outgrowth revealed a homogenous pattern of small, uniform cells with a cytoplasm-to-nucleus ratio of approximately 1:1 on intact (C) and EDTA-treated (D) AM, with the latter more uniform than the former. Cross-section analysis showed that most HLE on intact AM (E) migrated on top of the devitalized AM epithelial cells (*arrows*), whereas HLE on EDTA-treated AM (F) had direct contact with the amniotic basement membrane. Bar, (C, D) 100 μm; (E, F) 50 μm.

the cultures reached near confluence in 3 to 4 weeks. HLE cultures grown on EDTA-treated AM showed a significantly higher outgrowth rate than those on intact AM and reached almost confluent growth after 12 days ($P < 0.05$; Fig. 2). Cultures on EDTA-treated AM started to grow on day 2, whereas those on intact AM began to grow on day 4. The growth rate, measured by the slope of the curve, was identical for both culture conditions between days 4 and 8. However, the growth rate increased from days 8 to 12 for cultures on EDTA-treated AM, whereas that of the intact AM group showed a slight decrease.

Cx43 Expression and Cell Cycle Analysis

After 3 to 4 weeks of culturing on intact AM, the majority of expanded HLE did not express Cx43, regardless of the final outgrowth area's being 70% or 90% or completely confluent. HLE expressing Cx43 were calculated as 12.4 ± 14.5-positive units on intact AM, which was significantly lower than 57.5 ± 18.2-positive units of the EDTA-treated AM (Figs. 3A–C; $P < 0.05$). Positive Cx43 staining appeared in a punctate pattern, which was confined to the cell membrane of adjacent cells, compatible with the formation of gap junction channels (Fig.

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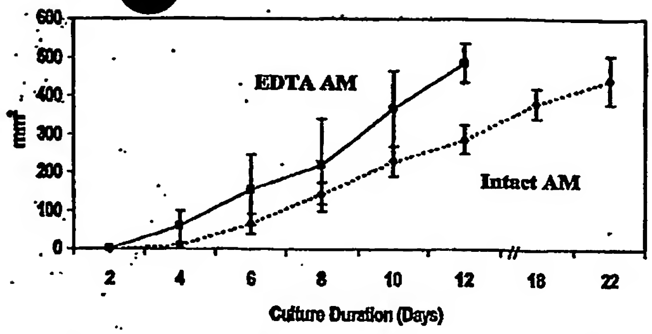

FIGURE 2. Outgrowth rate. A significantly faster growth rate was noted in EDTA-treated AM (*solid line*) than in intact AM (*dotted line*; $P < 0.05$). HLE on intact AM started to grow after 4 days compared with 2 days on EDTA-treated AM and reached final outgrowth after approximately 3 to 4 weeks compared with 2 weeks on EDTA-treated AM. Between days 4 and 8 both cultures grew at similar growth-rates. After 8 days the growth rate of EDTA-treated cultures increased slightly, whereas cultures on intact AM decreased slightly.

3B). In both groups Cx43 expression was found in localized areas predominantly adjacent to the explant or randomly scattered among the expanded cells without any preferred location.

To correlate Cx43 expression with the proliferative activity at the same time, we labeled the S phase of the cell cycle with BrdU, a thymidine analogue, for 24 hours in nearly confluent cultures, and performed double immunostaining. The labeling index was low, in the range of 2.4% ± 0.9% ($n = 5$) for the intact AM group (Fig. 4A), which was significantly lower than the 22.5% ± 8.2% ($n = 5$) in the EDTA-treated AM group (Fig. 4B; $P < 0.05$, Fig. 4C). Because the AM used in this study did not contain vital epithelium or fibroblasts, BrdU could have been incorporated only by expanded HLE cells. Areas with high BrdU uptake were found predominantly near the explant or at the leading edge of the outgrowth and were devoid of Cx43 expression (Fig. 4A, inset). To confirm that the nonlabeled cells were indeed slow cycling and not postmitotic differentiated cells, we continuously incubated a set of six cultures with BrdU for 6 days. As shown in Figure 4D, the BrdU-labeling index increased to 62% ± 9.5%. This result indicated that most of the expanded HLE cells on intact AM were indeed slow cycling. BrdU detection using immunostaining with FITC as a fluorescence probe further allowed visualizing HLE nuclei that incorporated BrdU (Figs. 4E, 4F; horizontal, open arrow) and amniotic epithelial cells (Fig. 4E, vertical arrow) at the same time. Expanded HLE growing on top of amniotic epithelial cells (Fig. 4E) showed an overall lower BrdU incorporation compared with areas where HLE expanded on EDTA-denuded AM had direct contact with the amniotic basement membrane (Fig. 4F).

Semiquantitative Dye Transfer Assay

To evaluate whether immunohistochemically detected Cx43 was indeed assembled into functioning gap junction channels, we performed a semiquantitative dye transfer assay using the previously described scrape-loading technique.[20] HLE cells expanded on intact AM did not show any dye transfer from the scraped area to the adjacent cells in most of the scrape lines performed (Figs. 5A, 5B). When semiquantitated, HLE cells cultured on intact AM showed dye transfer to adjacent cells in 17% of the total length of 18 scrape lines (three cultures and six scrapes per sample). When the same sample was subjected to subsequent immunostaining, we did not detect any Cx43 expression in these areas (Fig. 5C). However, in those areas that revealed focal GJIC (Figs. 5D, 5E), we noted positive expression of Cx43 (Fig. 5F, punctate staining). HLE cells expanded on EDTA-treated AM showed a similar result, except that we noted a slightly increase of communicating areas, amounting to 27% of the total length of 18 scrape lines ($P = 0.42$). As a positive control, we also scrape loaded the outgrowth of HLE grown on plastic and found 94% of the total length of 18 scrape lines showing dye transfer to adjacent cells (Figs. 5G, 5H). The positive GJIC correlated with positive expression of Cx43 of

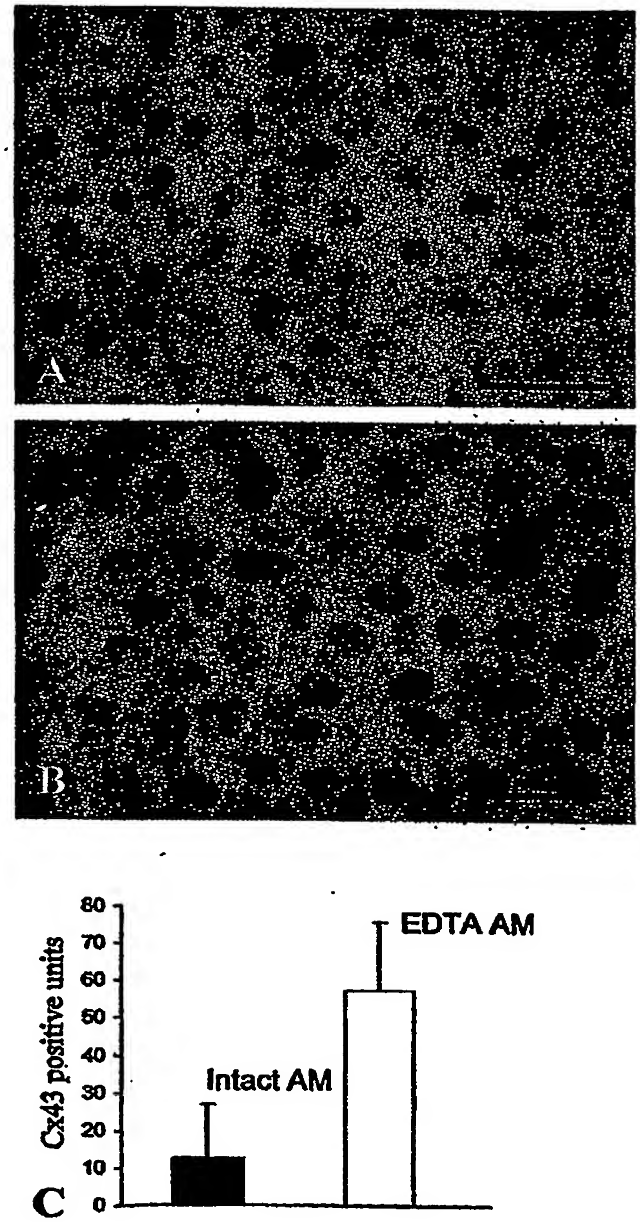

FIGURE 3. Cx43 expression. Most of the HLE cells on intact AM showed negative Cx43 expression (A), whereas more areas of HLE cells grown on EDTA-treated AM showed positive expression of Cx43 at the cell membrane (B). When semiquantitated, HLE cells on EDTA-treated AM expressed a significantly higher amount of Cx43-positive units than those on intact AM (C, $P < 0.05$). Bar, 50 μm.

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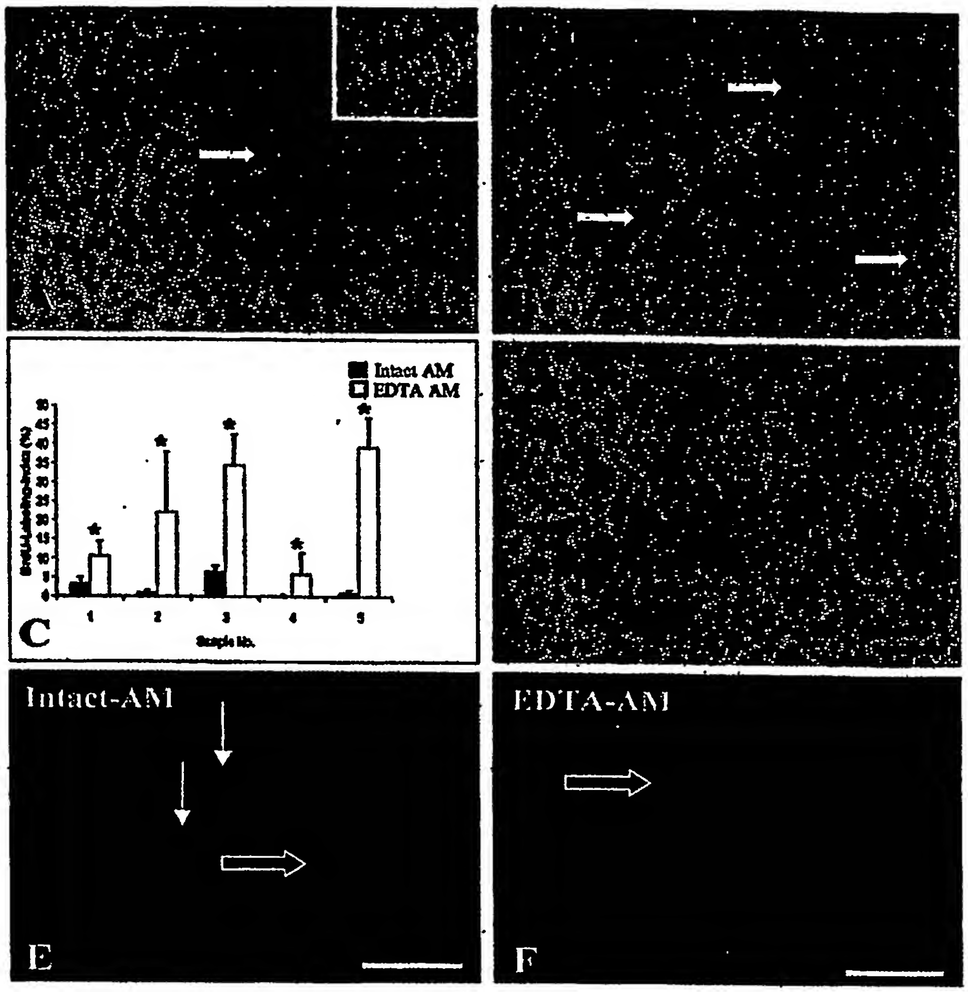

Figure 4. BrdU labeling. Twenty-four-hour labeling showed that a small fraction of HLE cells (*arrows*) had positively labeled nuclei on intact AM (A), whereas a large number of BrdU labeled nuclei were found in HLE cells on EDTA-treated AM (B, *arrows*, positive nuclei appear brownish). On intact AM only a few areas showed a high BrdU labeling index (A, *inset*). A significant difference in BrdU labeling was consistently noted in all five cultures analyzed (*P < 0.05; C). A 30-fold increase of BrdU labeling was noted when HLE cells on intact AM were continuously labeled for 6 days (D). Immunofluorescent staining of BrdU-labeled nuclei (*horizontal arrows*) was found in some HLE cells on intact AM that retained amniotic epithelial cells (E, *solid arrows*). In contrast, numerous BrdU-labeled nuclei were found in HLE on EDTA-treated AM without amniotic epithelial cells (F). Bar, 100 μm.

the same area (Fig. 5D). This amount of GJIC was significantly higher than amounts in both AM groups ($P = 0.002$).

Cx43 and K3 Keratin Expression and BrdU Retention after Xenotransplantation

To determine whether the increase of the BrdU-labeling index and Cx43 expression of HLE monolayer on EDTA-treated AM represents real phenotypic differentiation into TACs, we transplanted AM with expanded HLE as a composite graft ($n = 12$; 6 per group) into the subcutaneous plane of NIH bg-nu-xidBR mice to promote stratification and differentiation after the AM-cultured cells had been labeled continuously with BrdU for 7 days. In addition, we also looked for the expression of keratin K3, which is known to be positive in the suprabasal layers of the limbus and the full thickness of the corneal epithelium but is absent in the limbal basal epithelium.[1-4] Five days later, the resultant epithelium was stratified to an average of five cell layers in both groups. Basal cells were small and compact, whereas the more superficial cells appeared more flat and squamous (Figs. 6A, 6B). It should be noted that the existence of devitalized amniotic epithelial cells could not be discerned anymore as shown in Figure 6A. Expression of Cx43 was absent throughout the entire epithelium on intact AM (Fig. 6C, the inset shows the positive control of murine epidermis of the same specimen). Basal cells, but not suprabasal cells, on EDTA-treated AM expressed Cx43 punctate staining at the cell borders (Fig. 6D), resembling the phenotype of the corneal basal epithelium in vivo (Fig. 6D, inset). Expression of keratin K3 was absent in the basal layer on intact AM, but was expressed in the suprabasal and superficial layers of the same sample (Fig. 6E). In contrast, the stratified epithelium on EDTA-treated AM showed keratin K3 expression throughout all cell layers (Fig. 6F). BrdU label-retaining cells were detected in the basal layer in all samples of the intact AM group (6/6; Fig. 6G), but not in the EDTA-treated samples (0/6; Fig. 6H; $P = 0.0022$, Fisher exact test). Because the amniotic epithelium and fibroblasts were devitalized and showed no proliferative activity after the applied method of preservation,[18] the basal BrdU-labeled cells were judged to be derived from HLE cells.

Discussion

In this study we provided strong experimental evidence that the monolayer of HLE cells cultured on intact and EDTA-denuded AM expressed different phenotypes in Cx43 expression, GJIC, and proliferative activity. More Cx43 expression and a higher proliferative activity were promoted when HLE were grown in direct contact with the amniotic basement membrane (e.g., EDTA-denuded AM). After xenotransplantation to promote stratification and further differentiation, ex-

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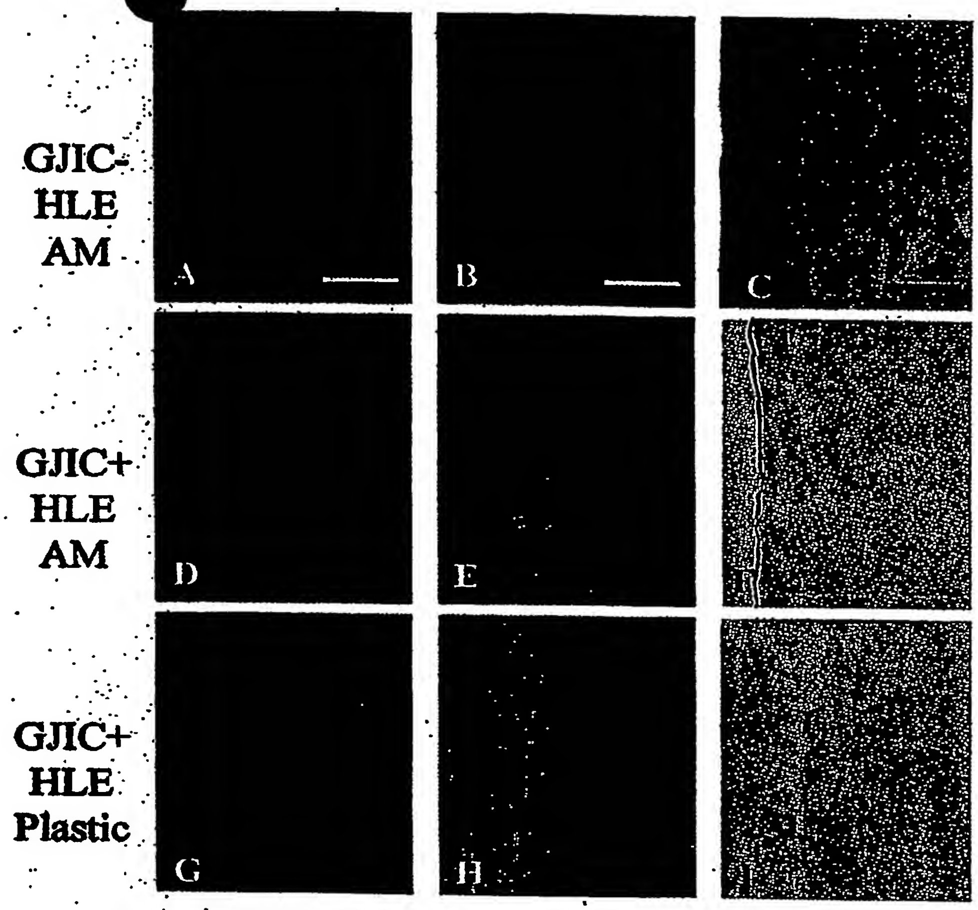

FIGURE 5. Scrape-loading dye transfer assay. Primary injured cells were identified by red fluorescence of rhodamine-dextran, which cannot be transferred to neighboring cells through gap junctions because of its high molecular weight. Communicating cells were identified by lucifer yellow, which can be transferred to neighboring cells through gap junctions. Using this method, most of the analyzed areas on intact AM showed no GJIC (A, B), when the corresponding area revealed no Cx43 expression by counterimmunostaining (C). Some localized areas of HLE cells on either intact or EDTA-treated AM showed GJIC (D, E). The corresponding area showed positive expression of Cx43 (F). In contrast, marked GJIC to adjacent cells was noted in the control of HLE cells cultured on plastic (G, H). The corresponding area showed positive Cx43 expression (I). Bar, (A, B, D, E, G, H) 100 μm; (C, F, I) 50 μm.

pansion on top of the devitalized amniotic epithelium (e.g., intact AM) promoted a Cx43-negative, keratin K3-negative and label-retaining basal epithelial phenotype, resembling that of the limbal basal epithelium in vivo. In contrast, EDTA-denuded AM promoted a Cx43-positive, keratin K3-positive, and nonlabeled basal epithelial phenotype, resembling that of the corneal basal epithelium in vivo. Collectively, these data support the hypothesis that intact AM preferentially preserves and expands limbal epithelial progenitor cells, whereas EDTA-denuded AM promotes corneal TAC differentiation.

Our data show that HLE expanded on intact AM had a significantly slower outgrowth rate than those expanded on EDTA-denuded AM (Fig. 2). This difference was due to a delayed onset of growth of cultures on intact AM and an increase of the growth rate on EDTA-treated AM in the late-culture phase. This finding was consistent with the data published by Koizumi et al.,[15] using rabbit limbal and corneal explants. Because 3T3 fibroblast feeder layers were used in their coculture system[15] but not in ours, we thus speculate that the fibroblast feeder layer does not play a major role in yielding such a difference in proliferative activity. One may speculate that such a different growth rate is caused by a mechanical resistance of a firmly adhered amniotic epithelium against expanding HLE that had to grow over them (Figs. 1E, 1F). However, another alternative explanation may be that HLE's proliferative activity was lower on intact AM, as shown by a significantly lower BrdU-labeling index on intact AM than on EDTA-denuded AM (Fig. 4). Koizumi et al.[15] as well as Meller et al.,[14] from our laboratory, demonstrated desmosomal structures between HLE and the underlying amniotic epithelium, using electron microscopy. We have recently conducted further analyses and noted that both integrins $\alpha6$ and $\alpha3$ were not expressed, whereas integrins $\beta4$ and $\beta1$ were expressed by HLE in contact with devitalized amniotic epithelial cells. This was in great contrast with the positive expression of integrins $\alpha6\beta4$ and $\alpha3\beta1$ by HLE when growing on denuded amniotic basement membrane (Grueterich et al., manuscript in preparation). This information strongly suggests that HLE uses different adhesion complexes when growing on intact versus EDTA-treated AM.

We further confirmed that the lower labeling index of HLE on intact AM was a result of a slow cell cycle and not of postmitotic differentiation, in that continuous BrdU labeling for 6 days caused a 30-fold increase in the labeling index (Fig. 4D). It should be noted that the BrdU labeling index was calculated at different time points, because both culture conditions grew at different growth rates. We naturally had the concern that, in cultured cells, proliferative activity slows when the cells approach confluence or plateau growth. However, we believe this concern would not have affected the interpretation of our data because the same growth condition was used for both groups during the labeling experiment. Immunofluorescent visualization of incorporated BrdU of HLE nuclei confirmed that the number of BrdU-positive nuclei was indeed higher in areas where amniotic epithelial cells were absent than in those where they were present (Fig. 4E, 4F). We thus conclude that HLE expanded on intact AM maintains a slower cell cycle—one feature of epithelial SCs in vivo. This

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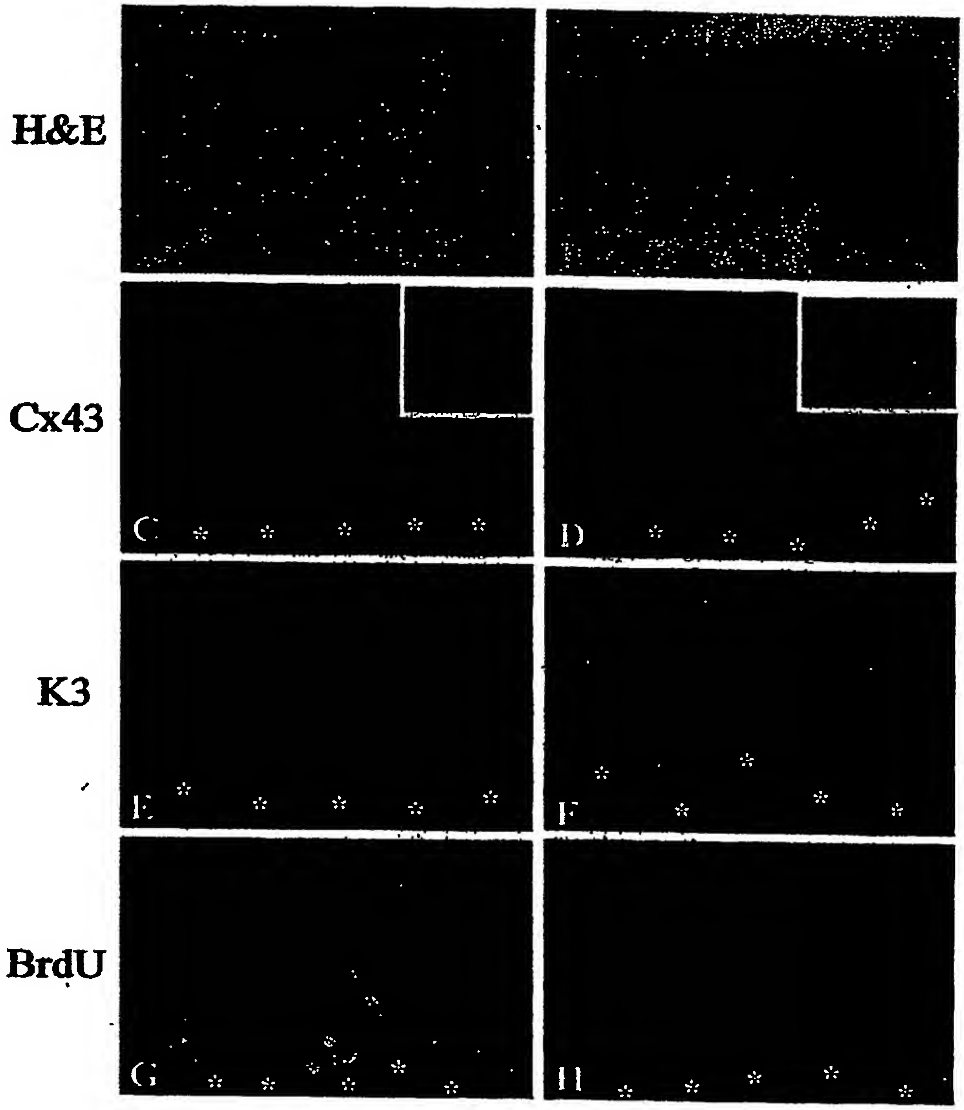

FIGURE 6. Phenotypes after xenotransplantation. Five days after subcutaneous transplantation, HLE on intact (A) and EDTA-treated AM (B) became stratified with relatively small cuboidal basal cells and a more flattened and squamous shape in the superficial layers. Cx43 expression was absent throughout all layers on intact AM (C), but positive in the basal layer in the EDTA-treated group (D), resembling the Cx43 expression pattern of corneal epithelium in vivo (D, *inset*). The positive control staining of Cx43 was found in mouse epidermis within the same sample that expressed abundant Cx43 (C, *inset*). K3 keratin expression was negative in the basal epithelium on intact AM (E), whereas positive in basal epithelium on EDTA-treated AM (F). When HLE cells were continuously labeled with BrdU for 7 days before xenotransplantation, BrdU-retaining cells could be exclusively identified in some areas of the basal layer (*green* fluorescence) on intact AM (G), but not in EDTA-treated AM cultures (H). The *red* fluorescence indicates nuclear staining with propidium iodide. * indicates the basement membrane. Bar, 100 μm.

finding was also consistent with previous findings by Meller et al. (Ref. 14 and manuscript submitted).

The next natural question is whether the rapid cell cycle and the higher outgrowth rate of HLE on EDTA-denuded AM represent a rapid self-renewal of limbal progenitor cells or their actual differentiation into TAC. Matic et al.[8] first proposed the theory that the absence of Cx43-containing gap junctions and GJIC is a mechanism by which SCs maintain their "stemness" in their specialized microenvironment, and expression of Cx43 activates GJIC that is needed for corneal TAC synchrony. We thus examined the expression of Cx43 and GJIC and noted that HLE cells expanded on intact AM were largely devoid of Cx43, a phenotype resembling that of the SC-containing basal limbal epithelium in vivo. Removal of the amniotic epithelium by EDTA mechanical debridement exposed the amniotic basement membrane and promoted the HLE to adopt a phenotype that showed significantly more Cx43 expression (Fig. 3). In both groups of AM, the patchy, positive GJIC was consistent with subsequent immunolocalization of Cx43 on the same samples (Fig. 5).

Positive GJIC areas of the EDTA-denuded AM group were larger than those of the intact AM group (27% vs 17%), but this difference was not statistically significant and was not consistent with the aforementioned 10-fold difference in Cx43 expression. There are several possible reasons for this discrepancy. First, Cx43 detected by immunostaining had not yet assembled into connexon associations between neighboring cells, which are essential for gap junction conductance. Second, it is not known whether gap junctions in corneal and limbal epithelium also form heterotypic junctions (i.e., a gap junction channel composed of different Cx subtypes). If this were the case, other Cxs might respond differently to the two culture conditions described herein, which may explain why we found no difference in GJIC, even though Cx43 expression was 10 times higher in cultures on EDTA-treated AM.

To further explore the cell population derived with our culture system, we transplanted expanded HLE on AM as a composite subcutaneous graft in immunocompromised mice to promote stratification and differentiation. In addition to the nude gene, which results in an absence of thymus-derived

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T-cells, these mice have two other mutations important in regulating the function of the immune system. They are X-linked immune defect (xid), which affects the maturation of T-independent B-lymphocytes, and beige (bg), in which the homozygote is devoid of natural killer cells that are cytotoxic to tumor cells in vitro. In both conditions a nicely stratified epithelium was found with a relatively small and compact basal cell layer. The presence of the devitalized amniotic epithelium could not be discerned underneath the stratified HLE any longer, suggesting their partial or complete disintegration, as proposed by others.[18] Our results showed that the basal layer of the resultant stratified epithelium on intact AM did not express Cx43, in contrast to HLE on EDTA-treated AM (Figs. 6C, 6D). In addition, we found that keratin K3 was absent in the basal layer on intact AM, whereas the basal layer in the EDTA-treated group expressed keratin K3 (Figs. 6E, 6F). Moreover, BrdU label-retaining nuclei were found in the basal layer of the epithelium on intact AM, but not at all in EDTA-treated AM cultures (Figs. 6G, 6H). That the amniotic epithelium and fibroblasts are devitalized and do not have any proliferative activity after the present method of preservation[18] shows that the basal BrdU-labeled cells are derived from HLE. Collectively, these data indicate that a rapid cell cycle and positive expression of Cx43 and keratin K3 were promoted when HLE grew directly on the amniotic basement membrane, strongly supporting the notion that this culture condition promotes TAC differentiation. This interpretation, however, cannot be extrapolated to the method used by Koizumi et al.[15,22] in which 3T3 fibroblasts feeder layers are routinely included. Nevertheless, in another report,[13] they mentioned that corneal differentiation is promoted, but failed to provide evidence that limbal epithelial progenitor cells are actually preserved. Future studies are needed to show that denudation of the amniotic epithelium to expose the amniotic basement membrane is an important microenvironmental "cue" in promoting TAC differentiation. This hypothesis can be tested by transplanting ex vivo expanded HLE in a rabbit model of limbal SC deficiency that we have recently reported.[23] If this were the case, we may understand why migration of the offspring of limbal SCs to the corneal basement membrane signifies the beginning of TAC differentiation. Future studies should also be extended to investigating which amniotic basement membrane component(s)—laminin-1, laminin-5, collagen VII, and fibronectin[24]—is responsible for upregulation of Cx43 expression and thus, TAC differentiation. In this regard, previous studies have shown that rat hepatocyte cultures[25,26] and human epidermal keratinocyte cultures[27] show an increase in gap junction synthesis and GJIC when exposed to certain extracellular matrix components (e.g., glycosaminoglycans, proteoglycans, and laminin-5).

Similarly, it seams equally important to delineate the role of devitalized amniotic epithelial cells in endowing HLE with a status of slow cycling and poor differentiation—that is, features resembling limbal epithelial SCs in vivo. Whether this role is a simple masking of the amniotic basement membrane or a release of cytokines from the devitalized amniotic epithelium, comparable to a feeder layer system, should be further investigated. Collectively, we believe the model system described herein is ideal for future exploration of the exact mechanistic operation in the microenvironmental niche that maintains "stemness" in limbal SCs, and in the cue that promotes corneal TAC differentiation. This new knowledge will help us understand the pathogenesis of limbal SC deficiency that develops in various ocular surface diseases and how transplantation of limbal epithelial SCs can be better refined in the future.

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